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**GENOTOXICITY OF 4-MONOCHLOROBIPHENYL IN THE LUNG OF
TRANSGENIC MALE 344 FISHER RATS**

by

Catherine Michael Maddox

A thesis submitted in partial fulfillment
of the requirements for the Master of Science degree
in Occupational and Environmental Health
in the Graduate College of
The University of Iowa

July 2007

Thesis Supervisors: Associate Professor G. Ludewig

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

Catherine Michael Maddox

has been approved by the Examining Committee
for the thesis requirement for the Master of Science degree
in Occupational and Environmental Health at the July 2007 graduation.

Thesis Committee: _____
Gabriele Ludewig, Thesis Supervisor

Larry Robertson

William Field

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CHAPTER I: INTRODUCTION AND STUDY HYPOTHESIS

1.1 Background on PCBs

Polychlorinated biphenyls are a class of organic compounds that were produced between 1929 and 1977 in the United States. An estimated two million tons of commercial PCB mixtures were produced world-wide during this period with roughly 0.2 million tons remaining in various environmental reservoirs (WHO 2003). These chemicals were used as coolants and lubricants, most commonly as dielectric fluids in transformers and capacitors. PCBs were also additives in paints, plastics, adhesives, and sealants. Most PCBs were sold as commercial mixtures of different congeners and sold in North America under the name Aroclor. Evidence of environmental accumulation resulted in a termination of production of PCBs in the United States in August of 1977 (ATSDR 2000). Today, evidence of PCBs remains even in remote regions, most likely as a result of atmospheric transport and precipitation (Vilanova 2001).

1.2 Exposure Pathways

There are a variety of ways that the general population can be exposed to PCBs. The three most common pathways will be discussed as well as the source of PCBs associated with each exposure type.

1.2.1 Oral or Ingestion

The major source of PCB in the body is from ingestion of contaminated food. Contaminated fish is the most common food that typically carries high levels of PCBs. The lipophilicity and stability of PCBs allow for their storage in adipose fat tissue and consequently biomagnification through the food chain. This bioconcentration is evidenced by the high levels of PCBs present in those animals near the top of the food chain. Also of concern for public health is that PCBs can be passed from mother to infant through breast milk. As a result, nursing mothers are usually advised to consume lower amount of fish compared to the general population. Two incidences of a small population ingesting a concentrated amount of PCBs have occurred, one in Japan known as the *Yusho* incident and the other in Taiwan known as the *Yu-Cheng* incident. Contaminated rice oil was the source of PCBs in both incidences. These exposed populations, as well as workers involved in PCB production, have been closely monitored to more precisely determine the effect of PCBs on humans.

1.2.2 Inhalation

Inhalation of PCB vapor particles results from contamination both indoors and outdoors. One common source for outdoor contamination seems to be vaporization from landfills containing PCBs, specifically from certain landfills listed on the National Priority List or as a Superfund site (Hansen and O'Keefe 1996; Hermanson and Hites 1989). Another outdoor source is vaporization from contaminated surface water (Hornbuckle and Green 2003). The main source of indoor contamination is vaporization of PCBs from construction material. A study by Hendrick et al. (2004) found that several

buildings surveyed in the Greater Boston area contained caulking material that exceeded the Environmental Protection Agency limit of fifty parts per million by weight. A study completed by Wilson et al (2001) found that for children, inhalation exposure was greater than ingestion exposure.

1.2.3 Dermal

Dermal exposure is the third way for humans to be exposed to PCBs but this exposure is largely an occupational hazard. Since production has been banned, this does not occur with any significant frequency. However, there are still electronic devices, like transformers and capacitors, that contain PCB mixtures and thus a small risk of dermal exposure still exists. Fortunately, studies have shown that washing the exposed skin with soap and water is effective in removing PCBs from the skin (ATSDR 2000).

1.3 Metabolism and Excretion

1.3.1 Metabolism

PCBs can be excreted unchanged but for the body to excrete a majority of PCBs, biotransformation must occur. The first step in biotransformation uses cytochrome P450 (CYP) enzymes to oxidize the PCBs to an arene oxide with an epoxide group. Arene oxides can undergo further biotransformation to a hydroxylated form with an OH group. The PCB used in this experiment was 4-Monochlorobiphenyl (PCB 3). The metabolic pathway for PCB 3 contains a hydroxylated intermediate or arene oxide intermediate which leads to a hydroxylated intermediate and then to a quinone (see Figure 1 for the

exact orientation) (Lehmann et al 2007). The arene oxide and quinone are electrophiles and can bind to nucleophilic macromolecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), hemoglobin and proteins (as summarized in Lehmann et al 2007). During this oxidative metabolism, reactive oxygen species can be generated and lead to DNA damage through strand breaks (Oakley et al 1996; Srinivasan et al 2001).

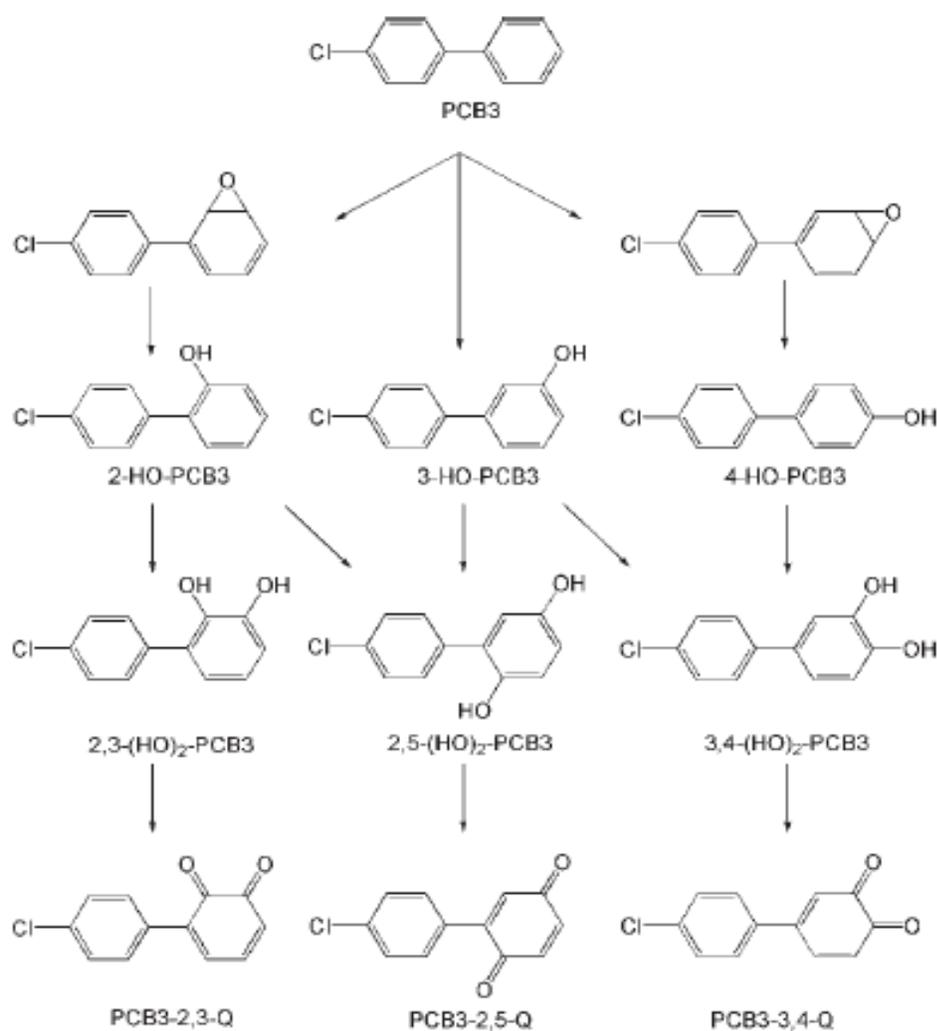


Figure 1: The metabolism of PCB3, including the formation of the arene oxide intermediates, the hydroxylated intermediate and finally the quinones (Lehmann et al 2007).

1.3.2 Excretion

For the most part, hydroxylated PCB metabolites are excreted in feces or urine or conjugated to sulfate or glucuronic acid (ATSDR 2000). Some metabolites are retained in the body due to reversible binding to proteins or the high lipophilicity. This leads to variability of retention times in the body. Studies have estimated half-lives ranging from 0.2 years to infinity, meaning no loss from the body (ATSDR 2000). A long half-life means that even after the source of PCB exposure is removed, there will still be a high concentration of PCBs in the body. Deposition of PCBs or PCB metabolites into adipose tissue could serve as a reservoir.

1.4 Health Effects

There are several health effects that are associated with PCB exposure. A brief overview will be provided for hepatic, endocrine, dermal, neurological, cardiovascular, and immunological effects. The focus of this study is on respiratory effects and thus more in depth information will be provided in that section.

1.4.1 Hepatic Effects

The most commonly studied health effect from PCB exposure is hepatic toxicity. There have been significant liver changes in animal studies but the results are far from conclusive (ATSDR 2000). Pathological changes seen in the livers of PCB exposed animals include deposition of fat in the liver, fibrosis of liver tissue, and necrosis. Induction of microsomal enzyme is commonly used in studies to examine the effect of PCBs since the enzyme levels are very sensitive to hepatic changes (ATSDR 2000). High

levels of microsomal enzymes such as cytochrome P450 exist in the liver and thus can provide insight to the effect of PCBs on enzyme levels. The induction of these enzymes can have a variety of effects on metabolism in the body, including changes in drug metabolism and an altered metabolism of endogenous compounds such as hormones. One clear example is vitamin A homeostasis in the body. PCB exposure can be manifested by the decreased storage of vitamin A in the liver. Cell growth and differentiation is dependent on Vitamin A. The exact mechanism of the effect of PCBs on vitamin A is unknown (ATSDR 2000). Animal studies have also shown that PCBs are hepatocarcinogens.

There are several hurdles in determining hepatic toxicity of PCBs in humans using epidemiological studies. One is inability to correct for the effect of confounding factors such as alcohol and age, which could affect liver health. Another is the possibility of misclassification of exposure levels. Nonetheless, based largely on the results of animal studies, the Environmental Protection Agency has classified PCBs as a probable human carcinogen.

1.4.2 Endocrine Effects

PCB exposure can affect the body by decreasing the levels of circulating thyroid hormones. Several animal studies have shown that PCBs passed either *transplacentally* or through breast milk can affect the thyroid hormone levels in newborn animals (ATSDR 2000). Thyroid hormones are very important during brain development. Data from human studies is limited, but an epidemiological study of people from the *Yu-Chen*

incident showed an increase risk for goiters in the adults. This increase may indicate a possibility of excessive thyroid disorders in the exposure population (ATSDR 2000).

1.4.3 Dermal Effects

Various dermal lesions can occur after PCB exposure. One of the most well known dermal reactions is chloracne. Chloracne is characterized by keratin plugs forming in hair follicles and sweat glands. Hyperpigmentation of the skin and nails has also been noted in humans following PCB exposure. These dermal effects were seen in occupational exposures as well as in people involved in the *Yusho* and *Yu-Cheng* incidents (ATSDR 2000).

1.4.4 Neurological Effects

Neurological effects of PCBs are highly studied in humans and animals. Results from human studies suggest that PCBs may cause subtle alterations in neural behavior in newborns and young children of mothers that possess a PCB burden. The long-term neurobehavioral effect is inconclusive since some studies have shown the alterations are still present more than a decade after *in utero* exposure and other studies have found the alterations are gone after two to four years (ATSDR 2000). The effect of PCBs on the central nervous system has been demonstrated through laboratory animal studies. The studies have examined both neural tasks and neurotransmitter levels in animals. Animals exposed to PCBs have shown a decreased performance in memory tasks as well as alterations that indicate impaired learning. The most consistent finding in studies of neurotransmitters is a decreased level of dopamine in various areas of the brain (ATSDR

2000). These findings do not allow for direct conclusions about neurotransmitter levels and neural behavior meaning the effect of lowered neurotransmitter levels in animals has not been studied.

1.4.5 Cardiovascular Effects

Many occupational studies have examined a possible link between PCB exposure and elevated blood pressure or other cardiovascular diseases. Unfortunately, results have been conflicting (ATSDR 2000). A study by Gustavsson and Hostedt (1997) found an increase in mortality due to cardiovascular disease in Swedish workers in a capacitor factory. Some of the *Yusho* population has shown an increase in triglycerides and total cholesterol level that were associated with PCB blood levels (Tokounaga et al 1999). Work done by Hennig et al (2002) suggests that PCBs may be involved in creating an inflammatory response which could be an underlying mechanism for atherosclerosis.

1.4.6 Immunological Effects

Studies of the *Yusho* and *Yu-Cheng* populations have provided that greatest insight into the immunological effects of PCBs on the human body. Studies using other populations are often affected by confounding factors that are difficult to control, specifically other toxic substances that could be immunotoxic as well. Studies in this area have shown consistently that the immune system has sensitivity to PCBs. The *Yusho* and *Yu-Cheng* populations have increased susceptibility to respiratory tract infections (ATSDR 2000). Results from animal studies include decreased antibody response and

reduced levels of T-lymphocytes. Gross alterations include thymic and splenic atrophy (ATSDR 2000).

1.5 PCBs and the Respiratory System

1.5.1 Respiratory Effects

The main source of information about the effect of PCBs on the respiratory system is epidemiologic studies of either occupationally exposed populations or from the *Yusho/Yu-Cheng* populations. A study completed by Warsaw et al (1979) found a decrease in vital capacity in exposed workers in a capacitor factory. Only one worker with restrictive spirometric impairment was found to have an abnormal roentgenogram. The results of a study by Fischbein et al (1979) found upper respiratory tract irritation, cough, and tightness of chest in workers from a capacitor factory. However, the prevalent use of volatile degreasers among the capacitor workers may be a confounder in this study (ATSDR 2000).

A study by Shigematsu et al in 1978 of four hundred *Yusho* patients found that many patients had bronchiolitis and few had pneumonia. Changes in roentgenograms, a type of X-ray, also indicated bronchiolitis in many patients. A long-term study of the *Yusho* patients was performed by Nakanishi et al (1985) to examine the respiratory status of those affected by PCB poisoning. This study involved both clinical data gathered over 11 years from *Yusho* patients starting one year after the exposure incident in 1968 as well as experimental data gathered from exposed male Sprague-Dawley rats. The researchers focused on the respiratory symptoms cough and sputa. Sputum is phlegm or mucus that is

expelled from the respiratory tract. Respiratory symptoms of the humans improved over the ten years of the study. Post mortem lung specimens were taken from seven individuals for pathologic examination. Infiltration of lymphocytes into the bronchial and bronchiolar walls as well as macrophage infiltration into the alveoli was seen. The rats in the experiment were dosed, sacrificed and then tissue prepared for microscopic examination. In the rat lung tissue slides, very mild pulmonary edema was seen.

Another respiratory effect seen in the *Yusho* population is more severe or more frequent respiratory infections (ATSDR 2000). The Nakanishi et al (1985) study examined the immune status of the *Yusho* population. Many of the *Yusho* population had decreased levels of IgA and IgM and increased IgG antibody levels soon after the exposure incident. The antibody levels returned to typical levels within a few years after the exposure.

1.5.2 PCB Deposition in the Lung

As mentioned previously, the liver and adipose tissue can be places of PCB deposition for short-term or long-term. Recent studies have shown that PCB deposition can occur in the lung as well. A study examining retention of PCBs in the blood of rats found that hydroxylated PCB metabolites were found in both the lung and liver and not in adipose tissue after fourteen days (Bergman et al 1994). The rats in that study were dosed orally not through inhalation exposure. Fouchecourt et al (1998) performed a study to examine levels of PCB in rats exposed through contaminated soil. The levels of PCBs in the lung after the inhalation exposure was 144 parts per billion, roughly 650-fold lower than the level of PCBs in the soil used in the experiment.

Work by Lund et al (1988) led to the purification of a PCB binding protein. This protein may explain the deposition of PCBs in the lung. The identification of this protein led to the inclusion of the lung in a study performed by Bachour et al (1998) that examined the species and organ dependence of PCBs in fish, foxes, roe deer and humans. The researchers found a high concentration of low-chlorinated biphenyls in the human lung. They concluded that the lung may be a target organ for accumulation of metabolically activated low-chlorinated biphenyls. These activated biphenyls can cause the formation of DNA adducts and subsequently DNA damage (Wong et al 1979; Dubois et al 1995).

1.5.3 PCBs as a Carcinogen in the Lung

As mentioned in the health effects section, PCBs are considered a probable human carcinogen mostly due to evidence from liver studies. Some studies have shown that PCBs exposure may lead to lung cancer as well. The review paper by Silberhorn et al (1990) suggested a role for PCBs as tumor promoters and further papers establish PCBs as a tumor promoter in the liver (Tharappel et al 2002). A paper by Nakanishi and Shigemastu (1991) reported that PCBs may act as tumor promoters by stimulating cell proliferation or inhibiting intracellular communication. A study performed by Anderson et al (1994) examined the tumor promoting activity of PCBs in the lung of mice. Mice were given N-nitrosodimethylamine (NDMA), a known cancer initiator, and then with Aroclor 1254 as a promoter. The mice were sacrificed at various time points and tumors quantified. There was a 2.5 fold increase in lung tumors in mice treated with NDMA as an initiator and Aroclor 1254 as a promoter compared to NDMA-only treated mice at 28

weeks. More recent studies have shown that PCBs are a complete carcinogen in rodents (Mayes et al 1998). Liver and thyroid cancer are the most commonly formed tumors in PCB exposed rats, but the rate of epitheliomas in the lung was also increased following exposure to various PCB congeners (NTP 2006a; NTP 2006b). PCB 3 has been shown to be mutagenic in the liver of rats (Lehmann et al. 2007). The researchers used the Big Blue © transgenic rats system to show the mutagenicity of PCB3. This is the same system used in this study as outlined in Chapter II.

1.6 The Big Blue® Assay

1.6.1 Overview

Transgenic assays provide researchers a way to assess tissue-specific mutations following *in vivo* treatment. The Big Blue ® transgenic assay is rodent based, with the ability to test the effects of mutagens in either mice or rats. The basis for this transgenic assay is the insertion of the *Escherichia coli lacI* gene as the λ LIZ shuttle vector into the rodent genome (Kohler et al 1991). A variety of exposure pathways and time points can be studied using transgenic rodents. Once the exposure period is over, the tissue of interest is harvested and the shuttle vector DNA is isolated. This DNA is then packaged into a lambda phage which will infect the *E. coli* bacterium and insert its DNA into the *E. coli* cell. The infected *E. coli* cells are plated on a selective medium to allow for the determination of mutants. See Figure 2 for a visual overview of the Big Blue assay. Portions of the transgenic assay used in these studies will be discussed more in depth.

1.6.2 Use of *lacI* Gene for Detection of Mutations

The use of the *lacI* gene allows for the Big Blue® assay to be selective. The *lac* operon gene is well studied and highly understood. The *lacI* gene codes for a protein that binds to the operator sequence known as *lacO*. The binding of the repressor protein prevents the binding of an RNA polymerase to the promoter region and thus prevents the transcription of several genes include the *lacZ* gene which codes for β -galactosidase. When there is not an inducer present, the repressor protein produced by the *lacI* gene will be bound to the operator region of the operon and thus the transcription of the *lacZ* gene are blocked. The removal of the repressor protein allows for the transcription of β -galactosidase. If there is a mutation in the *lacI* gene, the repressor is not made properly and thus the repressor protein is unable to bind properly to the operator region leading to the production of β -galactosidase.

There are two different selective plating methods to determine mutations. The colorimetric selection method detects changes in the *lacI* sequence by plating the *E. coli* on a medium that contains 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal), which is a chromogenic substrate. The protein β -galactosidase hydrolyzes the X-gal, and produces a blue color. The positive selection method involves plating the *E. coli* on a medium that has lactose as the sole carbon source. Only *E. coli* cells that produce β -galactosidase will grow on that medium because the lactose can be used as the carbon source. Plates using the positive selection method require a longer incubation period, up to 150 hours compared to the recommended 16 to 20 hours for the colorimetric system. The longer incubation period may lead to an increase in mutations derived from the *E. coli* and not from the treated rodent (Knoll et al. 1996).

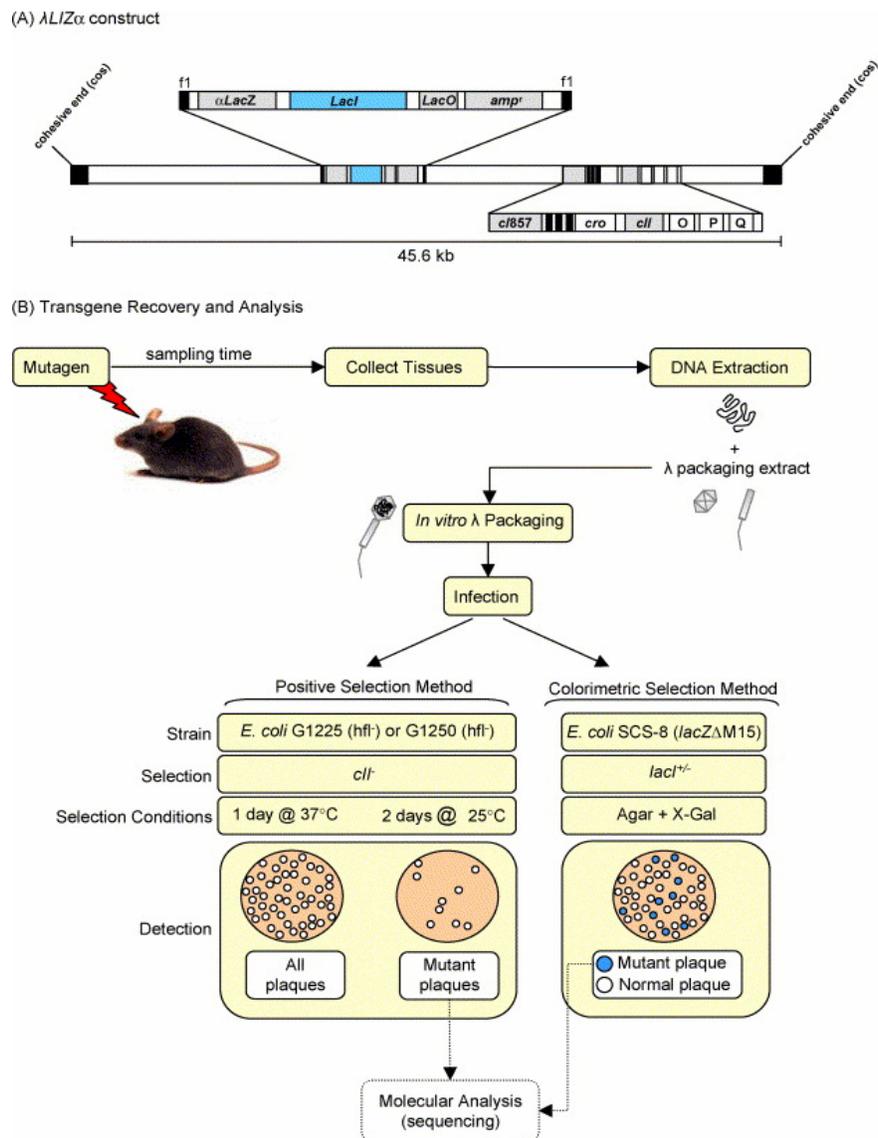


Figure 2: Overview of the Big Blue assay (Lambert et al. 2005). The colorimetric selection method was the method used in those studies outlined in Chapter II.

There are four distinct plaque color categories as defined by Stratagene for the colorimetric selection method. The control mutant CM0 is the lightest, then CM1, then CM2, and finally the darkest control mutant CM3. The control mutants were created as comparisons to help classify each mutant. The control mutants can also be used as

markers to determine when to remove plates from the incubator. The lighter colored control mutants, CM0 and CM1, can be plated every time to determine the amount of time required for the color to develop. Once the color has developed on the control plates, all plates can be removed from the incubator. Table 1 contains the location of mutation and the resulting amino acid change for each control mutant (Rogers et al 1995).

Control Mutant	Base Number	Mutation	Amino Acid Change
CM 0	530	C to T	Arginine to termination of protein
CM 1	179	C to A	Arginine to Serine
CM 2	381	G to A	Arginine to Histidine
CM 3	977	C to T	Glutamine to termination of protein

Table 1: Sequence and amino acid changes for each control mutant from Rogers et al 1995.

1.6.3 Creation of Transgenic Rodents

Mice were among first transgenic animal used commonly in toxicology studies (Kohler et al 1991a; Kohler et al 1991b). The transgenic mice contain the λ LIZ shuttle vector containing the *lacI* gene and the 5' portion of the *lacZ* gene. Transgenic rats were created from the 344 Fisher strain through a pronuclear injection technique (Dycaico et al 1994). The homozygous Fisher 344 strain has thirty to forty copies of the λ LIZ shuttle vector inserted on chromosome four. The rat strain was created because rats are more commonly used in toxicology studies. The larger organ size is preferred because of the amount of genomic DNA required for the packaging into the phage.

1.6.4 Dosing Recommendations

As mentioned previously, the Big Blue® assay can be used for a variety of exposure and treatment lengths. To mimic long-term exposure to environmental pollutants chronic or subchronic dosing should be used. Unfortunately, most laboratories do not have the resources for long term dosing. As a result, a short-term multi-dose protocol is a good compromise (Mirsalis et al 1995). The time between the last dose and sacrifice is known as the fixation. It is envisioned that during this fixation period, metabolic activation of the DNA-reactive form of the chemical is generated and DNA adducts are formed. The DNA adduct becomes a heritable mutation after one cell division cycle (Mirsalis et al 1995). The length of time required for this fixation time depends on the tissue type. Slowly dividing cells require a longer fixation time than highly proliferating tissue. If the fixation time is too long for highly proliferating tissue, the number of mutants can actually decrease due to apoptosis of those cells containing mutations (Mirsalis et al 1995). Heddle et al (2000) recommend a 35-day fixation when there are multiple tissues of interest.

1.6.5 Sample Size Recommendations

The sample size for each exposure group can greatly affect the ability to detect changes in mutation rates. Several studies have been done to determine the ideal sample size. Heddle et al (2000) recommend between five and ten animals for each treatment group. Callahan and Short (1995) recommend that ten animals be used if a 0.5 fold increase in mutant frequency is to be determined. Rogers et al (1995) recommend five animals per treatment group in order to measure a two-fold increase in mutant frequency.

Callahan and Short (1995) also recommend five animals per treatment except in the cases in which a strong mutagen is used as a positive control, in which a greater than two-fold increase in mutant frequency may occur. If this is the case, fewer animals may be used in the positive control group.

1.6.6 Plaque Plating Recommendations

Along with sample size, the number of plaques plated per animal affects the ability to detect increases in mutant frequency. If each animal within the treatment group is considered an experimental unit when determining the mutant frequency for the treatment group, there can be a great deal of variability within the treatment group. In an attempt to stabilize the mean and standard deviation within treatment groups, several studies have attempted to establish the ideal number of plaques to plate for each animal. Young et al (1995) found that plating 200,000 to 300,000 plaques per animal can stabilize the standard deviation within a treatment group. The mean mutant frequency stabilized when 100,000 to 500,000 plaques were plated. Young et al (1995) determined there was no appreciable improvement in detection of mutant frequency if more than 200,000 to 300,000 plaques were plated. Heddle et al (2000) found that between 125,000 and 300,000 plaques are ideal for determining a two-fold increase in mutant frequency. Callahan and Short (1995) recommended 300,000 plaques to be plated per animal.

The colorimetric selection method requires the identification of blue-ringed mutant plaques. The control mutant CM0 is very lightly colored and thus can be difficult to identify. Stratagene recommends using a red filter with a light-box to help identify the faintest mutant. One study has been carried out to improve the accuracy of mutant

identification (Bielas 2002). By changing the concentration of a buffer used, Bielas was able to darken the color of the faintest mutant. The darkened color allows for easier identification of the mutant plaques.

1.6.7 Mutant Frequency

The mutant frequency is defined as the number of mutant plaques divided by the total number of plaques plated for the entire treatment group. There have been several studies to examine the mutant frequency in untreated transgenic mice or rats. de Boer et al (1998) examined the mutant frequency in several tissue types in untreated mice. The highest mutant frequency was in the skin with the lowest mutant frequency in the bladder. The main way to determine untreated mutant frequency is to include control animals in the experiment as well as exposed animals. If a vehicle is required for the exposure pathway, for example corn oil is needed to dissolve PCBs, it would be ideal to treat the control animals with the vehicle only.

The age of the animals used in the study should be considered. Mirsalis et al (1995) found differences in mutant frequency between 3 week old mice and 6 week old mice. A later study by Stuart et al (2000) found no significant difference in the mutant frequency between 1.5 month old mice and 25 month old mice in the liver, bladder, or brain. These differences in mutant frequency will likely have a larger effect if the dosing regimen is long-term, for example 104-week subchronic studies, or if other tissues are studied that have higher proliferation rates, unlike the three tissues studied by Stuart et al (2000).

1.6.8 Mutation Spectra

The mutation spectrum seen in the Big Blue® assay reflects the types of DNA changes that are seen following exposure to a chemical mutagen. These changes are generally categorized as: transversion, transition, or frameshift mutations. Transversions occur when a purine is changed to a pyrimidine or a pyrimidine is changed to a purine. Adenine and guanine are purines and cytosine and thymine are pyrimidines. Transitions occur when one purine is changed to other purine or one pyrimidine is changed to the other pyrimidine. A frameshift mutation occurs when the number of bases that are deleted or inserted into the DNA strand is not divisible by three. The mutations are called frameshift mutation because the change in base number affects the reading frame during RNA to protein translation. It is believed that different treatments create different mutational events. This means that each specific mutagen can be correlated to a specific mutation spectrum (de Boer et al 1997).

A study completed by de Boer et al (1997) examined the mutation spectrum from the livers of untreated mice. The most commonly seen mutation was a base substitution, either a transversion or transition. The most commonly seen base substitution was a guanine to adenosine transition followed by guanine to thymine transversion. Frameshift mutations were much less common than the base substitutions. The deletion of one base was the most common frameshift mutation. Most of the frameshift mutations occurred in a four base repeating sequence of CTGG. Work completed by Kohler et al (1991b) led to the conclusion that this repeating base sequence is a mutational hotspot.

Areas of the DNA strand with high amounts of cytosine and guanine, known as CpG islands, are mutational hotspots for base substitutions. The most common mutation

in CpG islands is the transition of cytosine to thymine. This may be due to the deamination of 5-methylcytosine to thymine (Mirsalis et al 1995). Comparison of the mutation spectra from *E. coli* and transgenic mice provide the confidence that the mutations seen are mouse-derived and not *E. coli* derived (Hill et al 1998).

The packing reaction used in the Big Blue® Assay creates certain restrictions that may affect the ability of the assay to detect large deletions or insertions. This may lead to an inability to detect the genotoxicity of mutagens that cause clastogenic events. A clastogenic mutagen is able to cause breakages in chromosomes. The Big Blue® Assay is unable to measure changes at the chromosomal level and thus will not be able to detect large deletions or insertions that occur at that level.

1.7 Studies Utilizing the Big Blue® Assay

The Big Blue® assay can be used to assess mutagenicity of a variety substances and exposure pathways. A study completed by Rihn et al (2000a) established the mutagenicity of 3-methylcholanthrene (3-MC) in the livers of Big Blue® mice. There was a greater than 2-fold increase in the mutant frequency in the livers of treated mice. The mutation spectrum in the treated mice was mostly guanine to thymine and cytosine to adenine transversions. Shane et al (2000) found there was no increase in mutant frequency after mice were treated with benzo[a]pyrene, but changes in the mutant spectrum were seen. Treated mice showed elevated levels of guanine to thymine and guanine and cytosine transversions. The researchers concluded that benzo[a]pyrene may induce mutagenesis even though there is no statistical increase in mutant frequency. A

study of the breast cancer drug tamoxifen by Gamboa da Costa et al (2002) found an increase in mutant frequency in rats treated with tamoxifen and those treated with α -hydroxytamoxifen. Big Blue® mice were used to determine if vitamin E, as an antioxidant, will prevent oxidative DNA damage in vivo (Moore et al. 1999). Mice were fed either a control diet of 66 IU vitamin E or a high dose vitamin E diet of 1000 IU for 3 months. Five different tissue types were examined including heart, liver, adipose, thymus, and testis. The adipose tissue accumulated the highest levels of vitamin E and had the lowest mutant frequency. When the mutation spectra of all the tissues were pooled, there was a reduction in guanine to thymine transversions.

Big Blue® has been used to examine the mutagenic effect of γ - radiation near Chernobyl by Wickliffe et al (2003). Both female and male mice were kept in enclosures for 90 days in the Red Forest, which is 2 kilometers southwest of the Chernobyl Power Plant. There was not a significant difference in mutant frequency between the reference population and the exposed population. No significant changes in the mutation spectra were seen either population.

Big Blue® transgenic rodents show some promise of use in examining heritable mutations as shown by Barnett et al (2002). Male germ cells of Big Blue® mice were exposed to *N*-ethyl-*N*-nitrosourea (ENU), a known mutagen in Big Blue® mice. Ten weeks after exposure, the male mice were then bred to T stock females. The offspring were then screened using both somatic tissue and germ cells. The offspring mice were found to have identical mutations in the somatic cells and in germ cells. The researchers concluded that the results provided preliminary evidence that Big Blue® mice could be used to assess induced heritable mutations without creating an F₁ generation.

1.7.1 Big Blue® Assay Studies in the Lung

Big Blue® transgenic rodents have been used in several studies examining the mutagenic effect of certain chemicals in the lung. A study conducted by Muller et al (2004) examined DNA damage after oral exposure to diesel exhaust. The researchers were able to measure DNA strand breaks but there was not a significant increase in the mutant frequency in treated mice. Another study using diesel exhaust by Sato and Aoki (2002) found a 4.8 fold increase in the mutant frequency of exposed mice. The mutation spectrum was dominated by guanine to adenine and adenine to guanine transitions and guanine to thymine transversions. Asbestos fibers are another commonly studied mutagen in the lung. A study completed by Rihn et al (2000b) examined inhalation exposure to crocidolite fiber in Big Blue® mice. The mutant frequency increased in exposed mice compared to the control mice. There was no significant difference between the mutation spectra between the two groups. A study using amosite asbestos through oral exposure found an increase in mutant frequency after a 16 week exposure period in Big Blue® rats (Topinka et al 2004). There was no significant increase in mutant frequency after a four week exposure period. This led the researchers to theorize that the observed persistent inflammation and cell proliferation, as seen in wild-type 344 Fisher rats, may play a role in the mutagenic effect. Benzene exposure created a 1.8 fold increase in mutant frequency in the lung (Mullin et al. 1998). The exposure also created significant changes in the mutation spectrum, specifically an increase in the number of deletions and the number of bases deleted. These studies show that Big Blue® transgenic rodents can be used to assess mutagenicity in the lung effectively.

1.8 Study Hypothesis

The mutagenicity of PCB3 in the liver of male 344 Fisher transgenic rats has been established by Lehmann et al (2007). The goal of this study was to assess the mutagenicity of PCB3 in the lung of male 344 Fisher transgenic rats. The evidence that PCBs can be deposited in the lung and bound to a protein creates a need for research in this area. The study hypothesis is that PCB3 or its metabolite will increase the mutant frequency and change the mutation spectrum. This hypothesis was tested by treating Big Blue 334 Fisher transgenic rats with corn oil as a negative control, 3-MC as a positive control, PCB 3 and 4- OH- PCB 3. The rats were given intraperitoneal injections weekly for 4 weeks. Lungs were removed seventeen days after the last injection and analysis performed on mutation frequency, mutation spectrum, and pathological changes.

CHAPTER II: STUDY DESIGN

2.1 Materials and Methods

2.1.1 Chemical Substances

PCB3 and the hydroxylated metabolite, 4-OH-PCB 3 were synthesized, purified, and characterized as described by Espandiari et al. (2003). Agar and 3-MC were purchased from Sigma (St. Louis MO). 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-gal) was purchased from Research Products International (Prospect, IL). The DNA isolation RecoverEase kit and Transpack® kit were supplied by Stratagene (La Jolla, CA). All other chemical substances used were purchased from Fisher Scientific. Please see Appendix A for a complete description of the solution or media used in the study.

2.1.2 Exposure Protocol

The exposure protocol used was as described by Lehmann et al (2007). Briefly, a total of sixteen male Fisher 334 Big Blue® rats were purchased from Stratagene (La Jolla CA) and received from the breeder Taconic Laboratories (Germantown NY) at postnatal day 30. The Big Blue® rats are homozygous for the *lacI* transgene. Animals were provided with 7013-NIH-13 Modified Open Formula Rat diet and water ad libitum. The animals were kept on a 12-hour light/dark cycle. The animals were acclimatized for one week and then weighed prior to distribution into four exposure groups with four animals per exposure group. All animals received a weekly intraperitoneal injection. For the

positive control, 3-MC was used and a dosage rate of eighty milligrams per kilogram of body weight. Corn oil served as the negative control with the dosage rate of five milliliters per kilogram of body weight. The dosage rate of PCB3 was 113 milligrams per kilogram of body weight. The dosage rate of 4-HO PCB 3 was 82 milligrams per kilogram. The doses of PCB 3 and 4-HO PCB 3 were based on previous studies (Espandiari, et al. 2003; Espandiari et al. 2004). During the entire exposure period, animals were monitored daily to assess well-being and weighed twice a week. The last injection was given on postnatal day fifty-eight; seventeen days later all animals were euthanized by carbon dioxide followed by cervical dislocation. Lungs were excised and frozen in liquid nitrogen and then stored at -80°C until use. The right lobe was used to prepare histological slides at the University of Iowa Pathology Department and the left lobe was used for DNA isolation. For each animal four micron sections were cut, fixed on glass slides and stained with a hematoxylin and eosin stain. An Olympus BX40 light microscope was used for the examination of the slides. All experiments were conducted with the approval of the University of Iowa Institutional Animal Care and Use Committee.

2.1.3 DNA Extraction

DNA was extracted from the lung tissue using the Big Blue ® RecoverEase DNA isolation kit (Stratagene). Each extraction run included one animal from each exposure group used to prevent bias. 175 milligrams of lung tissue were taken from the left lobe of each animal. Due to the fibrous nature of the lung, each piece of lung was minced before being put into the tissue grinder. Prior to the addition of the lung tissue, five milliliters of

ice-cold lysis buffer, provided in the kit, was placed into the Dounce tissue homogenizer. The tissue was then added to the grinder. Using the “loose” pestle, the tissue was ground using between ten and fifteen strokes. Then the “tight” pestle was used for eight strokes without twisting the pestle to prevent shearing the genomic DNA. The homogenate was then poured through a cell strainer into a fifty milliliter tube and three more milliliters of lysis buffer were added. The entire process was performed at four degrees Celsius. The nuclei were then pelleted by centrifuging at 1100g for twelve minutes. RNAase and ProteinaseK were added to the pellet to digest the RNA and proteins. The solution was incubated at 50 °C for 45 minutes and then applied to a dialysis filter and dialyzed in TE buffer for forty-eight hours. The DNA was then removed from the filter using a wide-bore pipet tip and stored at 4°C.

2.1.4 *E. coli* Cultures

The *E. coli* strain SCS-8 was used as the host strain as recommended by Stratagene. The *E. coli* strain was purchased from Stratagene and stored at -80° C as a glycerol stock. Small plates of SCS-8 were made using a sterile inoculating loop to scrape the glycerol stock and then streaking a LB-tetracycline agar plate. The plate was then placed into a 37°C incubator overnight. A liquid culture was created by placing 20ml of NZY medium in a sterile 50ml conical tube to which was added 250 microliters of twenty percent (weight by volume) maltose and 1 M magnesium sulfate (MgSO₄). The *E. coli* bacteria were incubated for 4-6 hours in a shaking water bath at 37°C shaking at roughly 250 rpm. The tube was then centrifuged at 1000 g for ten minutes to pellet the cells. The supernatant was discarded and ten milliliters of 10 nM magnesium sulfate

(MgSO₄) was added to the tube to resuspend the pellet. The optical density at 600 nanometers was determined using a spectrometer and the culture was diluted to an optical density of 0.5 before use in the assay.

2.1.5 Packaging

The Lambda Phage Transpack® packaging kit was used to recover the lambda transgenic shuttle vector that contains the *lacI* target sequence. For each Transpack® reaction, a minimum of eight microliters and maximum of ten microliters were used. Each DNA sample was transpackaged and subsequently plated a minimum of three times. Every time a sample was transpackaged, two separate reactions using the same DNA were carried out and then the packaged DNA samples were pooled for plating. After the completion of the Transpack® reaction, a microcentrifuge tube contained the *lacI* transgene packaged into phages. The Transpack® reaction is diluted to one milliliter with SM buffer and then fifty microliters of chloroform is added and stored at 4° Celsius until used. The packaging efficiency was determined by infecting two hundred microliters of SCS-8 *E. coli* strain with one microliter of the Transpack® reaction and incubating the mixture for fifteen minutes. Three milliliters of top agar were added to the tube and plated on one hundred mm NZY-agar (bottom agar) plates. The plates were incubated at 37°C overnight. Bacteria that are infected by the phage create clear plaques on the lawn of bacteria. Plaques are counted and used to determine the number of 500 mm plates that needed to be poured. The number of plaques is multiplied by 1.3 then divided by 12,500 as outlined in the manual. The resulting number is equivalent to the

correct number of plates to be poured to identify mutants in the DNA sample. Please see Appendix B for the specific plaque number for each animal.

2.1.6 Plating

Two days prior to plating, roughly two hundred milliliters of bottom agar was poured into 500 mm assay trays (Corning). Trays are poured in advance to allow for evaporation of moisture. Excessive water can result in the hydrolysis of the 5-bromo-4-chloro-3-indolyl β -D- galactopyranoside (X-gal) without association with a mutant colony. For each plate poured, two milliliters of SCS-8 was required to create the lawn on the plates. Nine hundred microliters of the DNA package sample was added to the SCS-8 suspension and incubated for fifteen minutes. This is referred to as the SCS-8/DNA mixture. Two milliliters of SCS-8 suspension is added to control DNA and incubated as well. Two titer plates are poured for each pooled packaged DNA sample. To create the titer plates, two milliliters of fresh SCS-8 suspension are placed into two fifty milliliters tubes. After the incubation period, fifty microliters of the SCS-8/DNA mixture were added to these tubes. Two milliliter aliquots were placed in fifty milliliters tubes corresponding the correct number of plates. Thirty-five milliliters of top agar containing 1.5mg/mL of X-gal, dissolved in an amount of DMF that was calculated by multiplying the amount of X-gal used in grams by 4, were poured into each tube and then the top agar was poured onto the bottom agar. Assay trays were vented for one hour to prevent excess moisture and then inverted and placed in a 37° incubator for sixteen to twenty hours. All colorless plaques on the titer plates were counted and used to determine the number of plaque forming units (PFU) on each plate. Plates were examined for mutant plaques with

a blue phenotype. Mutant plaques were cored using a wide-bore pipette tip and then placed in a microcentrifuge tube containing five hundred microliters of SM buffer and fifty microliters of chloroform. Mutants were stored at 4°C to allow for the phage to elute from the agar. One microliter was taken from each microcentrifuge tube added to a fresh microcentrifuge tube containing 150 microliters of SM buffers. Two hundred microliters of SCS-8 suspension was incubated with 1.5 microliters of the solution for fifteen minutes. The solution was then plated in the same manner as described previously except using 100 mm² trays and five milliliters of X-gal containing top agar. The plates were incubated for sixteen to twenty hours and then examined for mutant blue plaques. One mutant per plate was cored and re-plated once more in the same manner. Any mutant that did not appear after the first re-plating at a lower density was removed from consideration as a mutant. Any mutants that were identified after the second re-plating at a lower density were cored and placed in 500 microliters of SM buffer and 50 microliters of chloroform and then stored at 4°C overnight. The next day, 400 ml of the SM buffer was removed while avoiding the chloroform phase and placed in a cryogenic tube with 30 microliters of DMSO and stored at -80° C for long-term storage. Mutant frequency was calculated by dividing the number of verified mutants by the total number of plaques as calculated from titer plates.

2.1.7 Amplification and Sequencing

To establish the mutation spectra, all verified mutants were amplified using the polymerase chain reaction and sequenced by the University of Iowa Sequencing Facility. Two primers were used during the amplification as outlined in the instruction manual,

one forward primer (5'-GTATTACCGCCATGCFATACTAG-3') and one reverse primer (5'-CGTAATCATGGTCATAGCTG-3'). The amplification program, as described in Lehmann et al (2007), was as follows: 30 amplification cycles at 94°C for 30 seconds, 53° C for 50 seconds, and 72°C for 60 seconds. Each PCR reaction contained 22 microliters of water, 25 microliters of Taq Hot Start (Quiagen), one microliter of forward primer, one microliter of reverse primer, and one microliter of the mutant to serve as the template.

Once the amplification process was completed, each PCR product was purified using a PCR Purification Kit (Quiagen). For each mutant, 30 microliters of PCR product were used for the kit. The purification process involved mixing 30 microliters of the PCR product with 150 microliters of PE buffer then applying the mixture to a microcentrifuge column. The tube was centrifuged for 55 seconds at 17,000 *g* to bind the DNA to the column. After discarding the flow through, 500 microliter of PB was applied to the column and spun for the same time and speed. The flow-through was again discarded and another 500 microliters of the buffer was applied to the column and spun again. The flow-through was discarded and the tube was spun for an additional minute at the same speed. The final step was to elute the DNA from the column; this was done by applying 80 microliters of Nanopure water to the column and letting the tube sit for two to three minutes before spinning the tube for one minute. The DNA from the purification was stored at 4°C. Once the PCR product was purified, it was run on a 17 mm 1% agarose gel to determine if the amplification worked. A 1 kbp DNA ladder was run on the gel to serve as a size reference. Each sample run on the gel contained the following: 7 microliters of SYBR Green ©, 3 microliters of the purified DNA, and 2 microliters of

loading dye. Each well contained 10 microliters and was run for 50 minutes at 85 volts. The size of the target sequence was roughly 1000 bp and therefore all PCR products had to show a band near that size before they would be sequenced. Each sample was checked using a spectrometer set at 260 and 280 nanometers to determine the purity of the DNA before submission to the sequencing facility.

The samples were submitted in a 96 well plate format. Each mutant was sequenced using four primers: forward PCR primer, #5 primer (5'-TCTGGTCGCATTGGGTC-3'), reverse PCR primer, #12 primer (5'-AGAACTTAATGGGCCCG-3'). Each well contained 2 microliters of a specific primer and 3.5 microliters of the DNA sample. The DNA sample used for sequencing was 182.8 ng of DNA dissolved in 20 microliters of Nanopure water. The raw sequence, both the forward and reverse sequences, was sent from the facility and each sequence was edited to remove the overlap of the forward primer and 5 primer. Basepairs upstream of the start codon and downstream of the stop codon were removed as well. The removal of the overlap and the basepairs outside the coding region created the final forward sequence. The final reverse sequence was created in the same fashion using the reverse primer sequence and #12 primer sequence. Each final sequence was compared with an established *E. coli lacI* sequence using the BLAST2 Sequence function accessed through the National Center for Biotechnology Information website. Both the forward and reverse sequences were checked using the BLAST2 Sequence function. Any disagreements between the sequences led to the re-sequencing of the mutant. Also any sequencing problems including an unidentifiable nucleotide within the coding region of the sequence resulted in re-sequencing of the mutant. Each mutant was then classified as a transition,

transversion, insertion, or deletion. Any insertion or deletion was classified as a frameshift mutation if the total number of basepairs inserted or deleted was not divisible by three. If any mutant sequence was identical to another mutant from the same animal, one mutant was removed from analysis. If any mutant sequence was identical to another mutant from a different animal, both mutants were kept in the analysis. Multiple mutations in the sequence for a single animal were counted as individual mutants.

2.1.8 Statistical Methods

A Student t-test was used to determine if there was a significant difference between the mean lung weight for the control group and the treatment groups. A Poisson regression analysis was performed to assess if there was a significant increase in the mutant frequency in the treatment groups.

CHAPTER III: RESULTS

3.1 Results

3.1.1 Lung Weights

Lungs from all animals were removed and weighed to determine any statistical differences between the control and exposed animals. Table 2 contains the weight in grams of the lungs from each animal and the treatment group as well as the average and the standard deviation.

Animal Number	Treatment Group	Lung Weight (g)
I 1	Corn Oil	1.190
I 2	Corn Oil	1.167
I 3	Corn Oil	1.062
I 4	Corn Oil	1.200
Average:		1.155 ± 0.063
II 1	3-MC	1.206
II 2	3-MC	0.827
II 3	3-MC	1.038
II 4	3-MC	0.636
Average:		0.927 ± 0.248
III 1	PCB-3	0.600
III 2	PCB-3	1.067
III 3	PCB-3	0.900
III 4	PCB-3	1.156
Average:		0.931 ± 0.245
IV 1	4-HO-PCB-3	1.232
IV 2	4-HO-PCB-3	1.018
IV 3	4-HO-PCB-3	1.168
IV 4	4-HO-PCB-3	0.681
Average:		1.025 ± 0.246

Table 2: Lung weights for each animal and the average and standard deviation for each treatment group.

3.1.2 Pathology Results

One lobe of the each animal lung was sent to the University of Iowa Pathology Department for slide preparation and diagnosis. Slides were treated with a hematoxylin and eosin stain. Changes seen in the lungs are shown in Tables 3-6, with each animal categorized into the correct treatment groups.

Treatment Group I: Corn Oil	
Animal Number	Pathological Changes
1	One focus of acute inflammation
2	Focal acute inflammation with focal increase in perivascular lymphocytes
3	Minimal perivascular lymphocytes
4	Minimal perivascular lymphocytes

Table 3: Pathological changes in lung tissue of animals from treatment group I.

Treatment Group II: 3 MC	
Animal Number	Pathological Changes
1	No abnormality
2	Focal perivascular lymphocytes
3	Focal perivascular lymphocytes
4	Focal perivascular lymphocytes with increased intra-alveolar macrophages.

Table 4: Pathological changes in lung tissues of animals from treatment group II.

Treatment Group III: PCB-3	
Animal Number	Pathological Changes
1	Increased perivascular lymphocytes
2	Peribronchial lymphocytes
3	Peribronchial lymphocytes with increased intra-alveolar macrophages.
4	Peribronchial lymphocytes

Table 5: Pathological changes in lung tissue of animals from treatment group III.

Treatment Group IV: 4-OH-PCB-3	
Animal Number	Pathological Changes
1	No abnormality
2	No abnormality
3	Minimal perivascular lymphocytes
4	Minimal perivascular lymphocytes

Table 6: Pathological changes in lung tissue of animals from treatment group IV.

The most common pathological change was perivascular lymphocytes, which is an accumulation in lymphocytes near veins in the lung. Lymphocytes are white blood cells that are important in the immune response. Intra-alveolar macrophages, macrophages in the alveola of the lung, were commonly seen as well. Macrophages are white blood cells that phagocytize foreign particles. Like lymphocytes, macrophages are involved in the immune response.

3.1.3 Transpack® Efficiency

The number of plaques observed after plating the packaged sample at a low volume were recorded and used to determine the number of bottom agar plates required as outlined in the Materials and Methods section. Please see Appendix B for the specific Transpack® efficiency for each animal.

3.1.4 Mutant Frequency

Mutant plaques were identified, verified and then sequenced. Prior to sequencing, the mutants were considered to be “uncorrected mutants” and used to calculate the “uncorrected” mutant frequency. Any identical mutants from the same animals were removed from the total numbers of mutants. The total number of mutants after

sequencing was used to calculate the “corrected” mutant frequency. The total number of plaques plated and corrected mutant number for each animal is listed in Appendix C. The mutant frequency was calculated for each exposure group on the basis of 100,000 plaques plated. The mutant frequency was calculated on the basis of 100,000 plaque rate to correct for variability in the number of plaques plated. The mutation frequencies for both “uncorrected” and “corrected” mutant numbers for each exposure group were calculated and shown in Table 7. The total number of corrected mutants for each animal and the total number of plaques plated for each animal are listed in Table 7.

Treatment Group	Uncorrected Mutant Number	Number of Plaques Plated	Per 100, 000 Rate (Uncorrected)	Corrected Mutant Number	Per 100,000 Rate (Corrected)
I	5	455840	1.096876097	4	0.877500878
II	43	342570	12.55217912	45	13.1360014
III	11	528220	2.082465639	10	1.893150581
IV	14	660420	2.119863117	12	1.817025529

Table 7: The uncorrected and corrected mutant numbers, plaques plated and mutant frequency for each treatment group.

3.1.5 Mutation Spectra

The sequencing of the mutants allowed for the determination of the mutation spectra. Table 8 contains the mutant name, the location of the mutation, the bases affected by the mutation, and the type of mutation. Appendix D contains the area of the sequence with the mutation site for each mutant.

Mutant Name	Site of Mutation	Basepair /Sequence Affected	Type of Mutation
I 1a	Bp 95	G to A	Transition
I 1b	Bp 136	G to T	Transversion
I 3a	After bp 322	Lose of 22 bp	Deletion/ Frameshift
I 4a	Bp 331	C to T	Transition
II 1a	Bp 199	G to C	Transversion
II 1b	Bp 487	C to T	Transition
II 1c	Bp 957	Loss of 1 G	Deletion/Frameshift
II 1d	Bp 202	G to T	Transversion
II 1e	Bp 272	C to T	Transition
II 1g	Bp 568	C to A	Transversion
II 1g	Bp 805	G to T	Transversion
II 1h	Bp 86	C to A	Transversion
II 1i	Bp 59	C to A	Transversion
II 1j	Bp 95	G to T	Transversion
II 1k	Bp 61	G to T	Transversion
II 1L	Bp 190	C to T	Transition
II 1m	After Bp 836	Loss of 1 G	Deletion/Frameshift
II 1n	Bp 845	G to T	Transversion
II 1o	Bp 192	A to C	Transversion
II 1p	Bp 180	C to A	Transversion
II 1q	Bp 608	C to A	Transversion
II 1r	Bp 530	C to G	Transversion
II 1s	Bp 152	C to A	Transversion
II 1t	Bp 182	G to C	Transversion
II 2a	Bp 152	C to A	Transversion
II 2b	Bp 526	G to C	Transversion
II 2c	After Bp 552	Loss of 1 G	Deletion/Frameshift
II 2d	Bp 94	C to A	Transversion
II 2e	Bp 88	C to T	Transition
II 2f	Bp 744	Loss of 1 C	Deletion/Frameshift
II 2g	Bp 464	C to T	Transition
II 2g	Bp 530	C to A	Transversion
II 2h	Bp 836	C to A	Transversion
II 2i	Bp 272	C to A	Transversion
II 2j	Bp 68	C to A	Transversion
II 2k	Bp 221	T to A	Transversion
II 2L	Bp 97	G to A	Transition
II 2m	After Bp 554	Loss of 1 C	Deletion/Frameshift
II 2o	Bp 223	G to T	Transversion
II 2p	Bp 919	G to A	Transition

Table 8: Mutant name, mutation site, exact mutation, and mutation type for each mutant sequenced

II 2p	Bp 926	C to A	Transversion
II 2q	Bp 180	C to A	Transversion
II 2r	Bp 331	C to T	Transition
II 2s	Bp 371	T to G	Transversion
II 2t	Bp 134	G to C	Transversion
II 2u	Bp 336	C to A	Transversion
II 2u	After Bp 337	Loss of 1 G	Deletion/Frameshift
II 2v	Bp 142	G to A	Transition
II 2w	Bp 95	G to T	Transversion
III 1a	Bp 193	C to G	Transversion
III 1c	After bp 246	Loss of 1 C	Deletion/Frameshift
III 2a	Bp 383	G to A	Transition
III 3a	Bp 787	G to C	Transversion
III 3b	Bp 142	G to T	Transversion
III 3c	Bp 92	C to G	Transversion
III 3d	Bp 190	C to T	Transition
III 3e	Bp 884	G to C	Transversion
III 3f	Bp 92	C to A	Transversion
III 3g	After bp 749	Loss of 1 T	Deletion/Frameshift
IV 1a	Bp 58	G to A	Transition
IV 1b	Bp 383	G to A	Transition
IV 2a	After bp 56	Loss of 1 C	Deletion/Frameshift
IV 2b	Bp 58	G to A	Transition
IV 3a	After bp 466	Loss of 31 bp	Deletion/Frameshift
IV 3a	Bp 615	A to C	Transversion
IV 3b	Bp 173	T to A	Transversion
IV 3c	Bp 180	C to A	Transversion
IV 3e	After bp 81	Loss of 1 C	Deletion/Frameshift
IV 3f	Bp 538	G to C	Transversion
IV 4a	Bp 44	C to T	Transition
IV 4b	Bp 272	C to T	Transition

Continuation of Table 8

The number of transversions, transitions and frameshift mutations were calculated for each group as well as the percentage of the mutation type. These values are shown in Table 9.

Exposure Group	Transitions	Transversions	Frameshifts	Total Mutants
I	2 (50%)	1 (25%)	1 (25%)	4
II	8 (18%)	31 (69%)	6 (13%)	45
III	2 (20%)	6 (60%)	2 (20%)	10
IV	5 (42%)	4 (33%)	4 (25%)	12

Table 9: The number and calculated percentages of transversions, transitions, and frameshift mutations for each exposure group.

The actual bases involved in the mutations were also determined. The number of each specific base change was determined for all treatment groups and is shown in Table 10.

Mutation	Group I	Group II	Group III	Group IV	Total
G to A	1	3	1	3	8
G to T	1	5	1	0	7
G to C	0	4	2	1	7
C to A	0	15	1	1	17
C to T	1	5	1	2	9
C to G	0	2	1	0	3
A to C	0	1	0	1	2
A to T	0	2	0	1	2
T to G	0	1	0	0	1
T to A	0	1	0	1	2

Table 10: Type of transversion and transition for each treatment group.

CHAPTER IV: ANALYSIS OF RESULTS

4.1 Analysis of Results

4.1.1 Lung Weight Differences

Determining the significance of differences in the means of the control and all three treatment groups were carried out by a Student t-test. The decrease in the average lung weight in the 3-MC treatment groups was not significant with a t-value of 0.19, with an α value of 0.05. The decrease in lung weight in the PCB 3 treatment group was not significant with a t-value of 0.16, with the sample alpha value. The decrease in lung weight in the OH-PCB 3 treatment group was also not significant with a t-value of 0.42. The variance within the treatment groups was greater than that of the control group. The exposure pathway may have an effect on the lung weight in terms of inflammation and cell proliferation. It is possible that an inhalation exposure may cause more inflammation and cell proliferation.

4.1.2 Pathological Changes

The pathological changes, specifically the increased levels of macrophages and lymphocytes may indicate inflammation in the lung due to PCB exposure. Topinka et al (2004) theorized that persistent inflammation and cell proliferation could play a role in the mutagenicity of amosite asbestos. It is possible that the level of inflammation is a function of the exposure route. The amount of inflammation may increase if inhalation exposure because more PCB particles could reach the lung.

4.1.3 Transpack® Efficiency

There was a great deal of variability in Transpack® efficiency; the number of plaques for each Transpack® reaction performed for each animal is shown Appendix B. Two of the animals in treatment group II had very low efficiency. The genomic DNA from animal 3 in treatment group III was not as viscous, and as a result not as concentrated, as other samples. Other animals with lower Transpack® efficiency had DNA samples as viscous as samples with higher efficiency. It is possible that there was contamination from proteins that did not dialyze from the DNA completely. The DNA in these samples could also have been sheared during DNA isolation. The Transpack® reaction requires that the genomic DNA be in one long strand. The fibrotic nature of the lung tissue required more force for proper homogenation of the tissue. This extra force requirement may have increased the likelihood of DNA being sheared during the isolation process.

4.1.4 Mutant Frequency

There were 72 total mutants identified and sequenced for all animals. There were 4 mutants in treatment group I, 45 mutants in treatment group II, 10 mutants in treatment group III, and 12 mutants in group IV. There was a 15 fold increase in the mutant frequency after treatment with 3MC. Treatment with PCB3 created a 2.2 fold increase in the mutant frequency. There was a 2.1 increase in mutant frequency after treatment with OH- PCB 3. A Poisson Regression analysis was performed using the mutant frequency. The results from the analysis are shown in Table 11.

Comparison	P-value
3MC vs. Corn Oil	2.17e-07
PCB 3 vs. Corn Oil	0.244
OH-PCB 3 vs. Corn Oil	0.208

Table 11: Results from the Poisson Regression analysis comparing mutant frequency.

The results from the statistical analysis show that the increase in the mutant frequency due to 3MC is statistically significant with a p value < 0.05 . The increase in the mutant frequency due to PCB 3 or OH-PCB 3 treatment was not statistically significant.

4.1.5 Mutation Spectrum

Base substitutions compose the majority of the mutations. Of the base substitutions, 69% of the mutations occurred in the first 400 basepairs. Transversions were the majority of the mutations overall, with 58% of all mutations being transversions and the most common type being a cytosine to adenine (24%). The next most common transversion was guanine to cytosine (10%) and guanine to thymine (10%). The least common transversion was thymine to guanine (1%). Transitions were the second most common mutation with 17% of all mutations being transitions. Cytosine to thymine (13%) and guanine to adenine (11%) transitions were the most common. The transition mutation of adenine to guanine and thymine to cytosine was not present at all. Deletions were the least common mutation with 17% of all mutations being deletions. A majority of the deletions were single basepair deletions (83%). Only 2 of all 12 deletions were deletions of multiple basepairs. There was no insertion mutations recovered. This does

not necessarily mean there were no insertion mutations following treatment. There are two possibilities why these mutations may not have been detected: restrictions of the phage packaging prevent the packaging of DNA that may have large insertions or deletions or the mutations are lightly colored that make the mutant plaques difficult to detect.

In treatment group I, 50% of the mutations were transitions, and transversions and deletions/frameshifts were both 25% of all mutations. Treatment group II had the highest number of transversions; with 69% of all mutations were transversions. Of the remaining mutations in treatment group II, 18% were transitions and 13% were frameshifts/deletions. The most common mutation in treatment group III was transversions (60%) and transitions and deletions were both 20% of all mutations within the treatment group. In treatment group IV, the most common mutation was transitions (42%). The second most common mutation in Treatment group IV was transversions (33%) and then deletions (25%).

Treatment group II and III had significant mutation spectra changes compared to the control group spectrum. Those two groups shifted to mainly transversions. The shift to transversions is less extreme in treatment group IV since the majority of mutations in group IV were transitions. The most transition in group IV was guanine to adenine transitions. In treatment group IV, there is only one more transition than transversions. The mutation spectra of the different treatment groups in the lung are similar to mutation spectra of the same groups described in the liver by Lehmann and coworkers (Lehmann et al 2007).

4.2 Conclusion

The purpose of this study was to determine the mutagenicity of PCB 3 or 4-OH-PCB 3 in the lung of transgenic Big Blue® Fisher 344 male rats. The lungs were removed and weighed to detect cell proliferation within the lung. Increased cell proliferation could cause an increase in mutation frequency since there is a possibility that more spontaneous mutations are created due to the high level of reproduction. However, if anything there was a decrease in the lung weight in Treatment groups II, III, and IV, but variability within the Treatment groups II, III, and IV was greater than the variability within the control group. The right lobe of the lung was sent to the pathologist to determine changes in the lung tissue. Only Treatment group III had consistent pathological changes. The most commonly seen pathological change was increased macrophages and lymphocytes. The presence of macrophages and lymphocytes indicates that there was an immune response in the lung. In animal I 1, there was an area of acute inflammation. No correlation of pathological changes and mutagenicity was observed. Treatment group II showed a ~15-fold increase in mutant frequency as well as a clear shift from primarily transitions to transversions, indicating that 3 MC is highly mutagenic in the rat lung. There was a two-fold increase in the mutant frequency following treatment with PCB 3 or 4-OH-PCB 3. Thus, even though the increase in mutant frequency was not statically significant, the treatment did create an increase in mutants. The difficulties with the Transpack® efficiency may have affected the detection of an increased mutant frequency following treatment. However, the mutation spectrum from treatment group III and IV are different from the control group spectra. This is similar to

result seen in the study by Shane et al (2002) which found no significant increase in mutant frequency following treatment with benzo[a]pyrene but found differences in the mutation spectrum in the exposed animals. Thus this study provides evidence that PCB 3 or 4-OH-PCB 3 may generate mutations in the lung following intraperitoneal exposure. More studies evaluating different exposure pathways may provide greater certainty of the mutagenic effect of PCB 3 and 4- OH-PCB 3 in lung tissue.

4.3 Recommendations for Future Studies

Future studies of the mutagenic effect of PCB on the lung can be done more efficiently and the results more conclusive if some changes are made to the study protocol. These changes can also make reproducibility easier. Recommendations for a variety of changes are made in the following section.

4.3.1 Animal Protocol

Several studies have recommending using 5 animals per exposure to determine a two-fold increase in mutant frequency (Callahan and Short 1995; Young et al. 1995). There were four animals used in the study, which is probably adequate. Future studies using Big Blue® rats should have 5 animals in each treatment group. A longer fixation time may be required to accurately detect mutations in slowly dividing tissue. A fixation period of 35 days after the last injection may be ideal if multiple tissues are going to be analyzed (Heddle et al 2000).

4.3.2 DNA Isolation and Transpack® Efficiency

Different tissues from transgenic rodents present some challenges to the some portions of the assay. The fibrotic nature of the lung tissue created some difficulty with DNA isolation. More strokes with the “loose” pestle were required to make the use of the “tight” pestle without causing damage to the mortar possible. Cutting the tissue into small pieces prior to placing it into the mortar helped, but did not completely eliminate the problem. These isolation problems could affect the Transpack® efficiency. There was high variability between and within treatment exposure groups in terms of Transpack® efficiency. One solution is to check the purity and concentration of the DNA spectrophotometrically at 260 and 260 nanometers as done by Topinka et al (2004). All DNA samples can then be diluted to same concentration to reduce variability. Checking the purity can determine if there are any contaminants that can be removed through purification or dialyzing the DNA again. An additional difficulty is that the RecoverEase DNA kit require ~0.175 grams of lung tissues, which is one lung lobe. Since one lobe was sent to the pathology department, only one DNA isolation for each animal could be performed. Thus if the DNA is sheared and it is difficult to get high Transpack® efficiency, a second DNA isolation is not possible.

A study by Bielas (2002) examined methods to increase Transpack® efficiency. Increasing the concentration of MgSO₄ from 10 mM to 0.5 M was found to increase the Transpack® efficiency two fold. This recommendation could be followed if there is difficulty in attaining high Transpack® efficiency.

4.3.3 Plaque Plating

The number of plaques plated greatly affects the ability to detect mutations as well as being a source of variability. Studies have established that 200,000 to 300,000 plaques should be plated to stabilize the mean and standard deviation (Callahan and Short 1995; Young et al 1995.) In order to be able to detect a two fold increase in mutant frequency as well as reduce variability, around 300,000 plaques should be plated. If the Transpack® efficiency for each animal can be increased and have similar efficiency, then plating 300,000 plaque would not lengthen the time required to complete the assay too much.

4.3.4 Other Exposure Pathways

As mentioned previously, the exposure pathway may affect the body's response to PCB3. A study using an exposure pathway would be usefully in assessing the effect of PCB3 or OH-PCB3 on the body. Studies have shown the PCBs will accumulate in the lung (Fouchecourt et al 1998; Bachour et al 1998). An inhalation exposure may lead to increase levels of PCBs in the lung and thus there could be a greater increase in mutant frequency.

APPENDIX A - SOLUTIONS

LB-Tetracycline Agar Plates

25 g Luria Broth

20 g Agar

Mix with 1 liter of Nanopure water

Autoclave for 30 minutes and cool to 55°C

Add 12.5mg of tetracycline (dissolved in 10ml of 50% ethanol and filter sterilized) and mix

Pour into sterile Petri dishes (100-mm) plate

Store plates at 4°C and protected from light

Bottom Agar

22 g NZY broth

15 g Agar

Mix with 1 liter of Nanopure water

Autoclave for 30 minutes

Mix briefly

Pour ~200 mL of the agar into 500 mm trays

Cure plates prior to use, Store plates at 4°C for 2 weeks

Top Agar

22 g NZY broth

6.8 g Agarose

Mix with 1 liter of Nanopure water

Autoclave for 30 minutes

Mix briefly

Pour into sterile 500 mL bottles

Store for up to a year

Maltose [20% (w/v)] and MgSO₄ (1M) Solution (per 100mL)

20 g maltose

24.6 g of MgSO₄ · 7 H₂O

Add Nanopure water to a final volume of 100mL

Filter sterilize

Store at 4° C for up to 6 months

10mM MgSO₄2.46 g MgSO₄ · 7 H₂O

Add Nanopure water to a final volume of 1 L

Autoclave for 30 minutes

Store for up to 1 year

SM buffer

5.8 g NaCl

2.0 g MgSO₄ · 7 H₂O

50 mL 1M Tris-HCl (pH 7.5)

5 mL 2% (w/v) gelatin

Add Nanopure water to a final volume of 1 L

Autoclave for 30 minutes

Store for up to 1 year

TE Buffer

10 mM Tris-HCl (pH 7.5)

1 mM EDTA

Autoclave for 30 minutes

Store for up to 1 year

APPENDIX B – TRANSPACK® EFFIECIENCY

Animal Number	Transpack Name							
	a	b	c	d	e	f	g	h
I 1	55	75	4	24	32	45	N/A	N/A
I 2	4	10	7	8	7	9	N/A	N/A
I 3	20	47	25	80	33	44	N/A	N/A
I 4	3	0	6	2	0	5	5	N/A
II 1	60	43	38	33	16	34	N/A	N/A
II 2	42	29	43	31	32	53	3	3
II 3	1	4	1	13	1	2	N/A	N/A
II 4	1	0	9	0	0	2	1	N/A
III 1	44	45	31	31	29	25	N/A	N/A
III 2	10	24	20	18	7	9	N/A	N/A
III 3	79	83	47	93	39	48	N/A	N/A
III 4	0	0	2	2	1	2	1	0
IV 1	45	86	34	46	45	49	N/A	N/A
IV2	7	1	5	1	1	1	N/A	N/A
IV 3	46	46	43	58	29	42	N/A	N/A
IV 4	43	35	35	20	19	26	N/A	N/A

In the table are the numbers of plaques for each Transpack® reaction performed. Table entries that say N/A were not performed because the efficiency was acceptable.

APPENDIX C: PLAQUE AND MUTANT NUMBERS FOR EACH ANIMAL

Animal Number	Plaques	Mutants
Treatment: Corn Oil		
I 1	173,700	2
I 2	3,330	0
I 3	278,240	1
I 4	600	1
Treatment : 3 MC		
II 1	173,640	20
II 2	164,870	25
II 3	3,800	0
II 4	780	0
Treatment: PCB3		
III 1	201,140	2
III 2	62,200	1
III 3	291,540	7
III 4	260	0
Treatment: OH-PCB3		
IV 1	273,920	2
IV 2	4,220	2
IV 3	248,000	6
IV 4	134,580	2

APPENDIX D: MUTANT SEQUENCES

The mutation in each sequence is bolded and italicized. If the mutation is a deletion the basepair upstream and the basepair downstream are both bolded and italicized. Mutants with multiple mutations have both mutation sequences listed.

I 1a:

TGTCTCTTATCAGACCGTTTCCCACGTGGTGAACCAGGCCAGCCACGTTTCTG

I 1b:

TCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGTAAAAAG

I 3a:

TCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTTCGAAGCCTGTAAAGCGG

I 4a:

TCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTTCGATGGTAGAATGAAGC

II 1a:

GCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACCTGCCGG

II 1b:

AGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACTAGACAC

II 1c:

AACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGCCAGGCGGTGAAGGGCA

II 1d:

GGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACCTGGCGTGCAAACAG

II 1e:

ACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGTGGCGATTAAATC

II 1g

CATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACA
TGGGCGCAATGCGCGCCATTACCTAGTCCGGGCTGCGCGTTGGTGCGGATAT

II 1h:

TGTCTCTTATCAGAACGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTG

II 1i:

GGGTGGTGAATGTGAAACCAGTAACGTTATACGATGTTCGAAGAGTATGCCGG

II 1j:

TGTCTCTTATCAGACCGTTTCCCCTCGTGGTGAACCAGGCCAGCCACGTTTCTG

II 1k:
GGGTGGTGAATGTGAAACCAGTAACGTTATACGATGTTCGCA**T**AGTATGCCGG

II 1L:
GCTGAATTACATTCCCAACCGCGTGGCATAACA**A**CTGGCGGGCAAACAGTCG

II 1m:
TACCGAGTCCGGGCTGCGCGTTGGTGC**G**GATATCT**C**GTAGTGGGATACGACG

II 1n:
TACCGAGTCCGGGCTGCGCGTTGGTGC**G**GATATCT**C**G**T**AGTGG**T**ATACGAC

II 1o:
TGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACA**C**CA

II 1p:
TGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAA**A**CGCGTGGCACAACA

II 1q:
AGCAAATCGCGCTGTTAGCGGGCC**A**T**T**AAGTTCTGTCT**A**GGCGCGTCTGCGT

II 1r:
CAACAGTATTATTTCTCCCATGAAGACGGT**A**GGCGACTGGGCGTGGAGCAT

II 1s:
TGGAAGCGG**A**GATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACA

II 1t:
TGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAAC**C**CGTGGCACAACA

II 2a:
TGGAAGCGG**A**GATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACA

II2b:
GATACCGAAGACAGCTCATGTTATATCCCGCCG**T**AACCACCATCAAACAGG

II2c:
CATGAAGACGGTACGCGACTGGGCGTGGAGCATCT**G**T**C**GCATTGGGTCACCA

II 2d:
TGTCTTTATCAGACCGTTTCCAGCGTGGTGAACCAGGCCAGCCACGTTTCTG

II2e:
TGTCTTTATCAGAC**C**TTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTG

II 2f:

ACAAACCATGCAAATGCTGAATGAGGGCATCGTT**CC**ACTGCGATGCTGGTTG

II 2g:

GCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGG**T**GTTATTTCTTGATGTCTC
TGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGACGGTA**AGC**

II 2h:

TACCGAGTCCGGGCTGCGCGTTGGTGCGGATATCT**AG**GTAGTGGGATACGAC

II 2i:

CCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCG**AG**GGCGATTAAATC

II 2j:

GGGTGGTGAATGTGAAACCAGTAACGTTATACGATGTCGCAGAGTATG**AC**GG

II 2k:

TTGCTGAATGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAAT

II 2L:

TGTCTTTATCAGACCGTTTCCCGCATGGTGAACCAGGCCAGCCACGTTTCTG

II 2m:

CATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGG**T**G**C**ATTGGGTCACCA

II 2o:

TTGCTGATT**T**GCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAAT

II 2p:

ATTTTCGCCTGCTG**AG**GGCAAAA**C**AGCGTGGACCGCTTGCTGCAACTCTCTCAG

II 2q:

TGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAA**A**CGCGTGGCACAACA

II 2r:

TCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGT**CG**ATGGTAGAA**T**GAAGC

II 2s:

CGGCGGTGCACAAT**CG**TCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTA

II 2 t:

TCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACG**CC**GGAAAAAG

II 2u:

GCCAGCGTGGTGGTGT**CG**ATGGTAGAACGAAG**AG**CGT**CG**AAGCCTGTAAAGC

II 2v:

TCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAA

II 2w:

TGTCTCTTATCAGACCGTTTCCCTCGTGGTGAACCAGGCCAGCCACGTTTCTG

III 1a:

TGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAAGA

III 1c:

TTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCTGCACGCGCCGTCGCAAATT

III 2a:

CGGCGGTGCACAATCTTCTCGCGCAACACGTCAGTGGGCTGATCATTA ACTAT

III 3a:

TGGGCCCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTTGGTGC GGATAT

III 3b:

TCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAT

III 3c:

TGTCTCTTATCAGACCGTTTGCCGCGTGGTGAACCAGGCCAGCCACGTTTCTG

III3d:

TGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCATAACA

III 3e:

TACCGAGTCCGGGCTGCGCGTTGGTGC GGATATCTCGGTAGTGC GATACGAC

III 3f:

TGTCTCTTATCAGACCGTTTACCGCGTGGTGAACCAGGCCAGCCACGTTTCTG

III 3g:

ACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCAACGCGATGCTGGTTG

IV 1a:

GGGTGGTGAATGTGAAACCAGTAACGTTATACGATGTCACAGAGTATGCCGG

IV 1b:

CGGCGGTGCACAATCTTCTCGCGCAACACGTCAGTGGGCTGATCATTA ACTAT

IV 2a:

GGGTGGTGAATGTGAAACCAGTAACGTTATACGATGTGCAGAGTATGCCGGT

IV 2b:
GGGTGGTGAATGTGAAACCAGTAACGTTATACGATGTCACAGAGTATGCCGG

IV 3a:
GCCTGCACTAATGTTCCGGCGTCAACAGTATTATTTTCTCCCATGAAGACGGT
CGTCTGGCTGGCTGGCATAAATATCTCCCTCGCAATCAAATTCAGCCGATAG

IV 3b:
CTGAATTACAATCCCAACCGCGTGGCACAACAACACTGGCGGGCAAACAGTCG

IV 3c:
GCTGAATTACATTCCCAAACGCGTGGCACAACAACACTGGCGGGCAAACAGTCG

IV 3e:
TGTCTTTATAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGC

IV 3f:
CAACAGTATTATTTTCTCCCATGAAGACGGTACGCGACTGCGCGTGGAGCAT

IV 4a:
GGGTGGTGAATGTGAAACCAGTAA TGTTATACGATGTCGCAGAGTATGCCGG

IV 4b:
ACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGTGGCGATTAAATC

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