CYTOCHROME P450 IN *DROSOPHILA MELANOGASTER*

CHARACTERIZATION OF CYTOCHROME P450

AND A

PUTATIVE CYTOCHROME P450 GENE

IN

DROSOPHILA MELANOGASTER

By

JANE CONSTANCE PURSEY, B.Sc.

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AUTHOR: Jane Constance Pursey, B.Sc. (University of Guelph)

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Abstract

Cytochrome P450 was examined in both insecticide resistant and insecticide susceptible strains of Drosophila melanogaster. Much higher levels were observed in the resistant strain IIID when compared to the susceptible strain Canton S. This increase appeared to be the result of an overproduction of a few existing forms. Two heme-staining microsomal proteins found in strain IIID were identified as putative cytochrome P450 isozymes. Polyclonal antibodies produced against these two proteins were used in the immunoanalysis of microsomal proteins from both strains.

A lambda gt11 cDNA expression vector library was created by inserting cDNA fragments from a Drosophila lambda gt10 library into lambda gt11 arms. The library was screened with the polyclonal antiserum. Three clones were isolated, of which one, gt11-A1, was most highly reactive with the antiserum. Analysis of the gt11-A1 lysogen indicated a 130 kd fusion protein was produced of which 16 kd was coded for by the cDNA insert. A .5 kb cDNA insert was isolated from the clone as part of a 1.5 kb KpnI/EcoRI fragment and was used in the analysis of Drosophila genomic DNA and total RNA. Southern analysis revealed an EcoRI polymorphism existed between strain IIID and Canton S. RNA analysis suggested strain IIID produced more coding message for the A1 insert in the larval and adult stages than did Canton S.

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Introduction

1.1 History of Insecticide Resistance

With the importance of agriculture to mankind, the protection of crops from physical damage has resulted in the development of a number of agricultural techniques. One of the most damaging effects on agriculture is brought about by insect pests which are capable of destroying entire crops. A means of controlling these pests had to be developed in order to save the crops from damage. Initial attempts at control were made using lime-sulphur treatments, however by 1897 the first observations were made that the codling moth and the San Jose scale were surviving these applications (Card, 1897; Smith, 1897). In 1908 it was observed that populations of the San Jose scale were resistant to the lime sulphur treatments (Melander, 1914). It was obvious that a more potent means of control would need to be developed. In 1945, shortly after the introduction of synthetic organic insecticides, DTT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane) was first used as a method of control of pests (Forgash, 1984). Two years later the first houseflies resistant to DTT were discovered (Georghiou, 1972). With the development of more potent pesticides and the more frequent application of them, the rate of development of resistance has increased dramatically. Prior to 1946 only one new resistant species was discovered every two to

five years. In the years immediately following the introduction of DTT (1946-1954) this rate increased to one to two per year. Since then the rate has been almost constant at seventeen new resistant species per year (Forgash, 1984).

One problem in the control of insect pests is that the insecticides used are not specific to pests only but also kill the natural parasites and predators of the pests (Georghiou, 1972). Because pest predator populations are dependent on host density the probability of predators developing resistance to insecticides is low. In fact, by the early 1970s, of 225 arthropod species resistant to insecticides only four were natural enemies of pests (Georghiou, 1972). By 1980 the total number of resistant species had increased to 428. The application of insecticides has had a cyclical effect. As more and stronger insecticides were applied, pest resistance increased, requiring higher dosages to be used. This in turn suppressed the population of the natural predators which resulted in an elevation of the pest population (Georghiou, 1972). In an attempt to combat these problems the initial reaction was to manufacture better and stronger pesticides. Perhaps however a better understanding of the mechanisms contributing to the insects' resistance would result in the development of a more effective means of control.

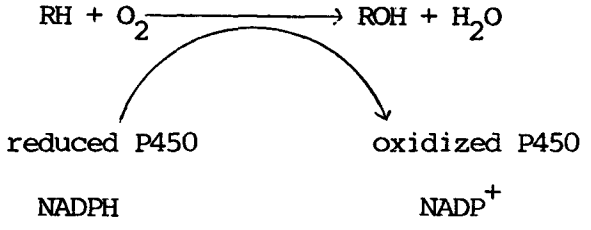
1.2 Cytochrome P450

The development of a metabolic means of dealing with foreign toxins probably occurred naturally in all herbivores, both arthropods and mammals. The plant food they ate contained a great number of toxic chemical compounds including phenolics, quinones, terpenoids and alkaloids (Brattsten and Wilkinson, 1977). The system used by these herbivores to detoxify these compounds entering their body is the basis of insecticide resistance. The enzyme systemn used is known as the mixed function oxidase (mfo) system, a system capable of both detoxifying and activating compounds. This system was initially discovered in mammals in 1958 independently by both Klingenberg and Garfinkel. Its presence in insects was first described by Ray (1965; 1967). One of the major components of the mixed function oxidase system is cytochrome P450.

Cytochrome P450 is a ubiquitous enzyme, having been isolated from various organisms including bacteria, yeast, plants, insects and mammals, and from various tissues in the mammal including kidney, lung, adrenal gland, colon, testis, brain, spleen, small intestine, muscle and ovary (Kaminsky et al, 1981). Most studies on cytochrome P450 however have been carried out on the mammalian liver.

1.2.1 Mode of Action of Cytochrome P450

Xenobiotics are foreign chemicals, often fat soluble which upon entering the body are not easily excreted unless they are made more polar. If not excreted they tend to accumulate in the organism causing toxic effects (Nebert et al, 1982). Two types of drug metabolizing enzymes have developed to convert these fat soluble compounds into water soluble compounds that are easily excreted. The enzymes are Phase I and Phase II drug metabolizing enzymes. Phase I enzymes introduce polar groups (eg. alcohols) into the parent molecule producing a substrate for phase II enzymes which attach more water soluble groups until the molecule is sufficiently polar so as to be excreted (Nebert et al, 1982). Cytochrome P450 is a phase I drug metabolizing enzyme which has mono-oxygenase activity. Its action is to insert one atom of molecular oxygen into the substrate in the following manner (Hodgson, 1985):



The second oxygen atom ends up in cellular water. During this process cytochrome P450 receives two electrons, one at a time, from NADPH (or occasionally NADH) via a cytochrome P450 reductase.

The ratio of cytochrome P450 to cytochrome P450 reductase content in the microsomal membranes ranges from 10:1 to 100:1 (Nebert and Gonzalez, 1987). The exact spatial arrangement of the two components is not known however given these ratios it has been suggested that cytochrome P450 molecules may be able to donate electrons to each other (Nebert et al, 1982). Studies do indicate the importance of the reductase molecule however, as mfo activity is inhibited when antibodies against the reductase are added to reconstituted mono-oxygenase systems (Hodgson, 1985).

Ironically the same mechanism that is employed by cytochrome P450 to detoxify compounds entering the body is also responsible for the activation of many precarcinogenic compounds (Nebert and Gonzalez, 1987). Experimentally this activation mechanism is commonly utilized in the Ames test (Ames et al, 1975) in which rat liver microsomes are observed to increase the mutagenic effect of chemicals and other compounds in the test organism Salmonella typhimurium. Similar work has been carried out using Drosophila as a eukaryotic test organism. It was in 1944 that Auerbach and Robson first used Drosophila to demonstrate the capacity of chemicals to induce mutations. Since then Magnusson and Ramel (1978) have used Drosophila extensively as a system for detecting mutations (specifically sex-linked recessive lethals, in which the target tissue is the gonads) caused by mutagens. Later Hällström et al (1981) found that Drosophila

microsomal fractions increased the rates of mutagenesis in the Ames test, suggesting that the mutagen activating compounds were present in the microsomes. Most environmental carcinogens are in fact activated by cytochrome P450 (Wolf, 1986) although insecticide resistance is an example of the rapid detoxification of a compound, preventing its toxic effects.

1.2.2 Characterization of Cytochrome P450

Cytochrome P450 is an integral membrane protein found in the microsomal fraction of cell homogenates, comprising as much as 3 to 12% of the total microsomal protein (Nebert et al, 1982). The microsomal fraction consists of all the cellular membranes pelleted from a post mitochondrial supernatant by high speed centrifugation. This includes smooth and rough endoplasmic reticulum and Golgi apparatus (Hodgson, 1985). The cytochrome P450 molecule is embedded in the membrane along with the P450 reductase which sits on top of the membrane. Much higher levels of mixed function oxidase activity have been found in the smooth endoplasmic reticulum when compared to the rough endoplasmic reticulum suggesting cytochrome P450 is preferentially located in the smooth membranes. When ethanol is used to induce cytochrome P450 in rat liver there is a proliferation of smooth endoplasmic reticulum, in addition to cytochrome P450 (Ko et al, 1987). Cytochrome P450 ranges in size from 43 - 60 kd based on SDS polyacrylamide gel electrophoresis (Nebert et al, 1982) although

one laboratory has reported isolating a 119 kd protein from Bacillus megaterium (Wen and Fulco, 1987). The active cytochrome P450 molecule includes a porphyrin ring containing iron that accounts for approximately 650 daltons that is lost during the denaturation processes required for SDS polyacrylamide gel electrophoresis. The heme combines with apoenzymes to form holoenzymes. The apoenzyme which ranges in size from 390-550 amino acids is responsible for the specificity to substrates (Nebert et al, 1982).

Cytochrome P450 is characterized as having an absorption maximum at or near 450 nm when reduced and treated with carbon monoxide. The carbon monoxide binds to the heme group giving it a characteristic difference spectrum. Occasionally a peak with an absorption maximum of 420 nm is found in microsomal preparations. This is a catalytically inactive form of cytochrome P450. Contamination by cytochrome P420 is more prevelant in insects than mammals, possibly due to proteolytic enzymes in the whole body or abdomen homogenates used (Hodgson et al, 1974). Interaction with other specific ligands affects the spectrum of cytochrome P450 causing characteristic maximums and minimums which are also useful in characterizing the molecule (Hodgson, 1985).

Cytochrome P450 is induced by many compounds, often substrates themselves. By 1967 more than 300 substrates were described as inducing their own metabolism (Nebert and Gonzalez,

1987). Other non-metabolized compounds such as ethanol, phenobarbitol and 3-methylcholanthrene are known to increase levels of cytochrome P450 in a variety of mammals and insects (Ko et al, 1987; Moldenke and Terriere, 1981; Nelson and Strobel, 1987). Naturally occuring substances in plants induce cytochrome P450 in the insects that feed on them. Brattsten and Wilkinson (1977) found that mixed function oxidase activity was induced in the larvae of the Southern armyworm when secondary plant products were added to their food. Similarily tobacco budworm larvae showed increased tolerence to insecticides after being fed wild tomato leaves (Riskallah et al, 1986). To be able to survive all the chemicals in the plants they eat insects must show a high degree of adaptability and versatility (Terriere, 1984).

1.2.3 Evolution of Cytochrome P450

The term "cytochrome P450" does not refer to a single protein but instead represents a superfamily of proteins capable of catalytic activity towards hundreds of different substrates (Nebert et al, 1982). This superfamily has a very ancient and divergent evolution. It is believed that all existing forms of cytochrome P450 originate from a single ancestral gene present more than 1.5 billion years ago (Nebert and Gonzalez, 1987). Researchers have generally agreed that gene conversion played a large role in the evolution of this protein. Cytochrome P450 is not only found in the endoplasmic reticulum but also in the

mitochondria. Based on the analysis of sequence data the mitochondrial cytochrome P450s more closely resemble prokaryotic cytochrome P450s than eukaryotic cytochrome P450s (Nebert and Gonzalez, 1987). This is consistent with the theory that mitochondria formed as a result of a symbiotic relationship between prokaryotes and eukaryotes. While microsomal cytochrome P450 is largely involved in detoxification and activation procedures, mitochondrial cytochrome P450 is more commonly involved in the biosynthesis of steroid hormones (Nelson and Strobel, 1987).

The cytochrome P450 superfamily has been divided into smaller groups based on the degree of relatedness between sequences (Whitlock, 1986). In the phylogenetic tree derived from the available data there are two major divisions, one being a division of eukaryotic versus prokaryotic families at 1400 Mybp, then at 900 Mybp a further division into endogenous versus drug metabolizing enzymes. At 400 Mybp the phenobarbitol family (a family induced by phenobarbitol) underwent rapid evolution. This was around the time that vertebrates first colonized the land. The evolution of plants on land several million years before vertebrates contributed to the diversity of the phenobarbitol family. During that time the plants developed toxic chemicals to which vertebrates had never been exposed. As a result the animals

were required to develop cytochrome P450 systems capable of handling the many different toxins (Nelson and Strobel, 1987).

1.3 Cytochrome P450 in Insects

Insects have developed a number of mechanisms to avoid the effects of insecticides. These include behavioural mechanisms such as avoidance, physiological mechanisms such as reduced penetrance of the insecticide due to an alteration in the makeup of the cuticle and biochemical mechanisms such as the development of cytochrome P450 (Hodgson, 1985). The detoxification ability of insects is expected to vary with strain, developmental stage and recent environment (Terriere, 1984). Although the first resistant houseflies were found in 1947, cytochrome P450 was not detected in insects until the mid 1960s by Ray. Until the early to mid 1980s cytochrome P450 research in insects had been carried out almost exclusively on the housefly (Terriere and Yu, 1979). The housefly cytochrome P450 system has been described as being as versatile as the mammalian liver system (Agosin, 1976). Other insects such as the fleshfly (Terriere and Yu, 1979), Southern armyworm (Brattsten and Wilkinson, 1977), blowfly (Terriere and Yu, 1979), tobacco budworm (Riskallah et al, 1986) and fruitfly (Naquira et al, 1980) have also been studied. Cytochrome P450 plays a number of important roles in insects in addition to the metabolism of xenobiotics. It is also involved in the metabolism of hormones and hormone analogues and the biosynthesis of phermones. Juvenile

hormone, important in the normal reproduction and development of insects is metabolized by cytochrome P450. Halpern and Morton (1987) have found decreased fitness in Drosophila strains with high cytochrome P450 levels.

Although Drosophila melanogaster is not considered to be an agricultural pest it is genetically well characterized and easy to maintain. Insecticide resistance has been studied extensively in Drosophila during the 1980s resulting in characterization of the genetic basis of resistance and some analysis of the proteins involved.

1.3.1 Mode of Action of Insecticides

The mechanism of action of certain types of insecticides has been determined. Organophosphates (such as malathion) and carbamates exert their effect on the enzyme acetylcholinesterase (Baillie and Wright, 1985). In addition, malathion also appears to have some inhibitory effects on succinoxidase, glycolysis and Krebs cycle intermediates (Dahm, 1971). Acetylcholinesterase catalyzes the hydrolysis of acetylcholine, a chemical neurotransmitter acting at synapses in the insects' central nervous system. When acetylcholinesterase is inhibited there is an accumulation of acetylcholine in the synapses, resulting in hyperexcitation. Insects exposed to carbamates and organophosphates exhibit initial hyperactivity, convulsions, paralysis and eventual death. Many organophosphates and

carbamates contain aromatic rings although it is not yet fully understood how they bind to the acetylcholinesterase molecule (Baillie and Wright, 1985). The relationship between acetycholinesterase activity and insecticide resistance has been examined. Morton and Singh (1982) found a negative correlation between activity and KT50 (the time required to knock down 50% of the flies) in isofemale lines exposed to the insecticide malathion. Between these lines they also found some variability in the electrophoretic mobility of the acetylcholinesterase enzyme. This suggested that these altered enzyme forms may have had a decreased affinity for the insecticide, reducing its effects. This is not however a cause and effect relationship, that is, the structural change in the acetylcholinesterase enzyme is not totally responsible for the increased resistance. Morton and Holwerda (1985) concluded that both the altered acetylcholinesterase molecule and increased mixed function oxidase activity contributed to the insecticide resistance.

1.3.2 Regulation of Cytochrome P450 in Insects

Cytochrome P450 in insects is found in the midgut, fat body and Malpighian tubules (Hodgson, 1983). Developmentally cytochrome P450 is very low or zero in the egg, then increases during each successive larval stage (although during the larval molts it is zero). During pupation and the pupal instar there is no activity. The activity is very low at emergence of the adult

fly, then rises to a maximum before decreasing again (Hodgson, 1983). Hällström et al (1983) found adult Drosophila had higher mfo activities than larvae possibly due to increased levels of digestive and proteolytic enzymes found in the larvae as a result of their eating habits. This type of difference has also been observed in foetal versus adult liver microsomes in mammals (Kato, 1979).

Induction of enzymes in insects was first observed by Agosin and Dinamarca in 1963 (Terriere, 1984). Induction of cytochrome P450 can be by plant products or manmade chemicals. In Drosophila, cytochrome P450 is induced by phenobarbitol and polychlorinated biphenyls (Hällström and Grafström, 1981). Upon SDS polyacrylamide gel analysis of housefly microsomal proteins, polypeptides normally observed only in resistant lines were also seen in susceptible lines that had been treated with phenobarbitol (Moldenke and Terriere, 1981). Often insects already having high cytochrome P450 levels do not produce significantly more cytochrome P450 when treated with inducers. For example in the Southern armyworm cytochrome P450 and associated activities are higher at 15^{o}C than at 30^{o}C, however at 30^{o}C more induction is observed (Brattsten et al, 1986). Hikone R, a resistant strain of Drosophila melanogaster which has relatively high levels of cytochrome P450 was not induced by phenobarbitol while nonresistant strains were (Hällström et al, 1982).

1.3.3 Insecticide Resistance in Drosophila melanogaster

Resistance to insecticides in Drosophila melanogaster has been studied extensively in the last ten years. The ability of Drosophila to metabolize chemical compounds has been demonstrated (Hällström and Grafström, 1981; Waters et al, 1982) and is correlated with increased mixed function oxidase activity (Morton and Holwerda, 1985). Hällström (1985) mapped some of the factors contributing to resistance to chromosome II (near 65) and chromosome III (near 51) (Hällström, 1985). This was substantiated by Houpt et al (1988) who suggested genes on chromosome II (at 64) and chromosome III (near 58) controlled malathion resistance. Many of the activities associated with resistance in insects are believed to be caused by a gene or genes called the RI locus (for resistance to insecticides) first described by Kikkawa in 1961 (Hällström and Blanck, 1985). Increased levels of certain microsomal proteins have been described in strains resistant to insecticides (Naquira et al, 1980; Hällström et al, 1984; Waters et al, 1984; Houpt et al, 1988). These proteins are believed to be cytochrome P450s.

1.4 Objectives of this Study

1.4.1 Background Information

In 1981, Singh and Morton began an investigation into insecticide resistance in Drosophila melanogaster. They collected natural populations from the Hamilton, Ontario area and made

isofemale lines from them. Forty of these lines were pooled and then separated into two control and two selected populations. The selected populations were exposed to either a high or low dose of the insecticide malathion in their food. Over a period of 110 generations a resistant population of flies was created from the line receiving the high dose of malathion. Halpern and Morton (1987) later made isochromosomal lines for each of the three major chromosomes from this resistant population. They determined there were genetic factors on both chromosome II and III that contributed to mixed function oxidase activity and decreased fertility. These factors were later mapped by Houpt et al (1988) to 2-64 and 3-58.

Previous work in this area has yielded an understanding of the genetics involved in insecticide resistance in Drosophila and some analysis of the proteins involved. No one yet however has isolated the gene coding for cytochrome P450 in Drosophila although monoclonal antibodies have recently been developed against different isozymes of Drosophila cytochrome P450 by Sundseth et al (1989) who have plans of screening an expression vector library for a gene. It would be interesting to determine how homologous the cytochrome P450 sequences from Drosophila may be to other cytochrome P450 sequences isolated from both mammals and insects.

1.4.2 Present Research

The purpose of this study was to examine cytochrome P450 in resistant and nonresistant strains of Drosophila melanogaster. After isolating cytochrome P450 from a resistant strain, antibodies produced against it would be used to screen a lambda gtll expression vector library. Lambda gtll is a system developed and modified by Young and Davis (1983a, 1983b). cDNA fragments are inserted into an EcoRl site near the 3' end of the β-galactosidase gene. When induced, β-galactosidase fusion proteins are produced which can be screened for antigenicity towards the antibody. Any clones obtained would then be used to characterize genomic DNA and total RNA from both resistant and susceptible Drosophila melanogaster strains.

Materials and Methods

2.1 Biochemicals

All biochemicals were of the highest quality available and unless specifically indicated were obtained from a major supply house.

2.2 Culture of *Drosophila melanogaster*

2.2.1 Strains of *Drosophila* Used

Two *Drosophilia melanogaster* strains were used in this study. Canton S, a wildtype strain susceptible to insecticide, was obtained from Dr. M. Kidwell (Department of Ecology and Evolutionary Biology, University of Arizona). Strain IIID is an isochromosomal strain for the third chromosome isolated and described by Halpern and Morton (1987). It was derived from a population selected for resistance to the insecticide malathion over 110 generations (Singh and Morton, 1981).

2.2.2 Maintainance of Flies

Flies were maintained on banana medium (10 g malt powder, 20 g sugar, 40 g yeast powder, 1 banana, and 12 g agar (Difco) per 1200 ml ddH_2O, with .2% p-hydroxybenzoic acid to inhibit fungal growth) at 24^{o}C on a 12 hour day/night cycle in 16 dram plastic vials or 8 oz glass culture jars. When large numbers of flies

were needed for microsomal preparations they were collected in a population cage (45 x 30 x 30 cm) and the adults were fed a mixture of dried yeast, sugar and water.

2.3 Analysis of Microsomes

2.3.1 Microsomal Preparation

Microsomes were prepared from susceptible and resistant strains using the method described by Houpt et al (1988). Flies were homogenized in TKEG buffer (50 mM Tris pH 7.4, 50 mM KCl, 1 mM EDTA, 10% glycerol) at a concentration of 4 ml buffer/g fly. The homogenates were centrifuged at 3000xg, 4°C, 5 minutes using an SW34 rotor in a Sorvall RC-2 centrifuge. The pellets were rehomogenized with a further 2 ml TKEG/g fly and recentrifuged. Particulate matter was removed from the pooled supernatants with two high speed centrifugations at 20,000xg, 4°C, 20 minutes. Microsomes were pelleted by centrifugation at 135,000xg for 30 minutes (SW50.1 rotor) or 45 minutes (SW27.1 rotor) in a Beckman L8-70 ultracentrifuge at 4°C after which they were homogenized in a glass tissue grinder on ice in buffer 2 (100 mM KPO_4 pH 7.25, 1 mM EDTA, 0.1 mM DTT, 20% glycerol; .4 ml/g fly). In most cases microsomal membranes were solubilized by adding CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane- sulfonate) (Sigma C3023) to a final concentration of 0.5% and grinding briefly with a tissue grinder. Insoluble material was removed

with a second ultracentrifuge spin at 135,000xg for 45 minutes. All samples were stored at -70°C.

2.3.2 Column Purification of Microsomes

Cytochrome P450 was partially purified from the CHAPS solubilized microsomes as described by Houpt et al (1988). At room temperature, CHAPS solubilized microsomes were applied to a 5 x 1 cm hydroxylapatite (Biorad Biogel HTP) column previously equilibrated with 10x buffer A (100 mM KPO_4, pH 7.25, 10 mM EDTA, 1 mM DTT, 20% glycerol) and 10x buffer A containing .5% CHAPS. The column was washed with 10x buffer A + .5% CHAPS and fifty drop fractions were collected on a Buchler Fracto-Mette 200 fraction collector. Those having a brown colour were pooled and designated fraction I. They were diluted two fold with 1x buffer A and passed through a long (20 x 1 cm) DEAE column equilibrated with 1x buffer A + .1% CHAPS. Protein was eluted off with 1x buffer A + .3% CHAPS + .175 M NaCl. Fractions with high absorbance at 420 nm were pooled (fraction II), diluted two fold with 1x buffer A and loaded onto a 5 x 1 cm octylamino-Sepharose-4B hydrophobic column equilibrated with 1x buffer A + .1% CHAPS. The octylamino column was prepared by attaching the ligand 1,8-diaminooctane (Sigma D2888) to cyanogen bromide activated Sepharose-4B. Sepharose-4B agarose beads (Sigma 4B-200) were activated with cyanogen bromide prior to attaching the ligand (adapted from Nishikawa and Bailon, 1975). After washing 2 ml packed non-activated Sepharose beads

with water and resuspending in a final volume of 6 ml ddH_2O, 3 ml cyanogen bromide solution (25 mg/ml in ddH_2O) was added and the reaction continued to completion (about 10 min) while the pH was maintained near 11 with 1N NaOH. The activated Sepharose was washed well with ice cold ddH_2O followed by coupling buffer (0.1M $NaHCO_3$ pH 8.0 + .5M NaCl). 1,8-diaminooctane was attached using methods described by Pharmacia (1979). The ligand (48 mg), dissolved in 15 ml coupling buffer, was combined with the activated Sepharose and incubated overnight at $4^{o}C$ with gentle agitation. The remaining active groups on the Sepharose were blocked at room temperature with 0.2 M glycine at pH 8.0 for 2 hours. Uncoupled ligand was removed by washing the gel alternately with high and low pH buffers (0.1 M $NaHCO_3$ pH 8.0 + 0.5 M NaCl or 0.1 M NaAcetate pH 4.0 + 0.5 M NaCl) ending with the pH 8.0 carbonate buffer. This octylamino-Sepharose-4B was used to purify the fractions from the DEAE column. Proteins were eluted off using a salt gradient of 0 to .5 M NaCl in 1x buffer A + .3% CHAPS. Again 420 nm absorbing fractions were collected, pooled and concentrated by dialysis against PEG 20,000 (fraction III).

2.3.3 Cytochrome P450 Content

Total cytochrome P450 content was determined by measuring the absorbance between 400 and 500 nm of a reduced sample through which carbon monoxide had been bubbled, as described by Estabrook

and Werringloer (1978). The base line of the spectrophotometer was determined by reducing CHAPS solubilized microsomal samples, diluted two fold with spectral buffer (300 mM KPO_4 pH 7.8, 50% glycerol), with sodium dithionite. After bubbling CO through the sample cuvet for one minute, the CO difference spectrum was measured. Cytochrome P450 content was calculated according to the Beer-Lambert law $A=\epsilon cl$. "A" is the difference in absorption between 490 nm and 450 nm, "l" is the path length (1 cm), "c" is the concentration of cytochrome P450 and "ϵ" is the CO difference extinction coefficient of cytochrome P450 (91 $mM^{-1}cm^{-1}$) (Jefcoate, 1978).

2.4 Protein Analysis

2.4.1 Protein Quantitation

Protein concentration was determined using the method of Lowry et al (1951) with slight modifications. Samples containing 5 - 50 µg protein were mixed with .5 ml water and 2.5 ml reagent 1 [49 ml of 2% Na_2CO_3 + 1 ml (.5% $CuSO_4(H_2O)_5$, 1.2% Na_3citrate)] and incubated at room temperature for 10 minutes. Folin reagent (Fisher 50-P-24, .25 ml of a 33% solution) was added and the absorbance at 660 nm was measured after one hour. Bovine serum albumin (Boehringer-Mannheim 238 031) was used to make a standard curve.

2.4.2 Electrophoretic Analysis of Proteins

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was used to analyse samples and to determine molecular weights of various proteins. The procedure followed was that of Laemmli (1970) as modified by Coulthart (1986). For general analysis 1.5 mm thick gels (16 x 14 cm) with a 10% resolution gel and a 4.8% stacking gel were used. Protein standards for molecular weight determination were bovine serum albumin (BSA) (68 kd, Boehringer Mannheim (BM) 238 031), beef liver catalase (58 kd, BM 106 810), pig heart fumarase (48 kd, BM 104 957), and bovine carbonic anhydrase (30 kd, BM 103 187). All gels were run at $4^{o}C$. Gels were electrophoresed at 80V for 16 hours and silverstained to detect protein according to the method of Morrissey (1981). Gels to be heme stained were 3 mm thick to facilitate loading of large volumes. E. coli lysates were separated on 5% resolution gels with 3% stacking gels at 150V for 6 hours with β-galactosidase (116 kd, Sigma G8511), phosphorylase b (97.4 kd, Sigma P4649) and BSA as molecular weight markers. With the exception of those to be heme stained, all samples were mixed with an equal volume of 2x sample buffer (125 mM Tris pH 6.8, 4% SDS, 10% β-mercapto ethanol, .01% bromophenol blue (BPB), 20% glycerol) and boiled three minutes prior to loading. Heme gel samples were mixed with 1/2 volume heme sample buffer (200 mM Tris

pH 6.8, 3% SDS, .01% BPB, 30% glycerol) and loaded directly onto the gel without heating.

2.4.3 Heme Staining

The method of Mesulam (1982) for histochemical heme staining was adapted for SDS polyacrylamide gels. Gels containing 300 µg samples of nondenatured microsomal proteins were washed in 10% methanol (10 min), 25% isopropanol in 0.1 M Tris pH 7.6 (2 x 15 min), and ddH_2O (15 min) after which they were preincubated in the dark for 20-30 minutes with 100 ml ddH_2O, 5 ml 0.25 M sodium acetate pH 5.0, 10 ml 1 mg/ml 3,3',5,5', tetramethylbenzidine (Sigma T2885) in methanol and 100 mg potassium ferrocyanide, heated gently to dissolve. Development occurred in the dark with the addition of 5 ml 0.2 M imidazole containing 3% H_2O_2. The resulting blue bands tended to fade quickly so it was important to photograph the gel immediately.

In order to stain for total protein after photographing, the gel was destained by washing 30 minutes in a 1% sodium sulfite solution in ddH_2O (Thomas et al, 1976a), then restained with Coomassie blue (1% in ddH_2O:methanol:acetic acid (5:5:1)) for 1 hour (Singh and Coulthart, 1982). The gel was destained overnight in the same solution minus the dye.

2.4.4 Western Blotting

SDS polyacylamide gels were electro-blotted onto nitrocellulose membrane (Scheicher and Schuell, BA85) using a Biorad transblot system. Gels were equilibrated in 25 mM Tris, 60 mM glycine buffer for 20 minutes at room temperature and sandwiched between Whatman 3MM filter paper with the membrane as described by Towbin et al (1979). Transfer was carried out with a current of .25A for four hours with stirring at 4°C in transfer buffer (25 mM Tris, 60 mM glycine in 20% methanol). The blotted nitrocellulose was blocked with a 5% solution of skim milk powder in water, air dried and stored between filter paper until probed. Amido black (.1% amido black in methanol:ddH_2O:acetic acid (9:9:2)) was used prior to blocking in order to stain for total protein (Towbin et al, 1979). After staining at room temperature for 3-5 min excess stain was washed off with ddH_2O.

2.4.5 Photography

Permanent records of gels and western blots were made with Kodak TMAX 135 mm film, ASA 100 using a Pentax 35 mm camera, 1/30 second exposure.

2.5 Immunoanalysis of Proteins

2.5.1 Production of Antibody Against Strain IIID Microsomal Proteins

Serum was collected from a male New Zealand white rabbit prior to injection with antigen to serve as a preimmune serum control. The rabbit was injected intracutaneously and intramuscularly with .8 ml (1.1 mg) CHAPS solubilized column purified microsomal proteins (fraction III) from the resistant strain IIID, emulsified with an equal volume of Freund's adjuvant. Four weeks after the initial injection the rabbit was boosted intramuscularly with .5 ml (.7 mg) of a similar sample minus the adjuvant. One week later 8 ml serum was collected and was designated the primary bleed serum or "Ab1". The rabbit was boosted a second time with .3 ml (1 mg) strain IIID microsomal proteins (fraction III) eight weeks after the initial injection and terminally bled 1 1/2 weeks later, collecting 120 ml serum (terminal bleed serum or "Ab2"). After collection all serum was allowed to clot at room temperature for several hours. Cooling of the serum to $4^{o}C$ facilitated removal of the clot. A short spin (3000 rpm 10 min) removed red blood cells. Sodium azide was added to .1% (w/v) and the undiluted serum was stored at either $4^{o}C$ or for a longer term at $-70^{o}C$ with no apparent decrease in activity in either case.

2.5.2 Purification of the IgG Fraction

The IgG fraction of the terminal bleed immune serum (Ab2) was purified by ammonium sulphate precipitation followed by DEAE column purification as described by Thomas et al (1976b). Proteins from 60 ml terminal bleed serum were precipitated overnight at 4°C by the addition of solid ammonium sulphate to 40% saturation with gentle stirring. Following centrifugation (10,000xg, 10 min) precipitated proteins were resuspended in 60 ml 50 mM KPO_4 pH 7.4 and were reprecipitated for one hour at 4°C with ammonium sulphate (35% saturation). After harvesting, proteins were dissolved in 12 ml 20 mM KPO_4 pH 8.0 and dialysed for several days against the same buffer at 4°C to remove any residual salt. Insoluble material was removed by two centrifugations (10,000xg, 10 min) and the supernatant was loaded onto a DEAE column equilibrated with 20 mM KPO_4 pH 8.0. Protein was eluted off the column with the same buffer and 200 drop fractions were collected. Those with significant protein, as determined by their absorbance at 280 nm, were pooled, constituting the IgG fraction (Ab2-IgG).

2.5.3 Production of Rabbit Antibodies Preferentially Directed to Strain IIID Microsomal Proteins

Strain IIID specific antibodies were prepared from the primary bleed serum (Ab1) by exposing serum to Canton S microsomal proteins bound to a Sepharose column. Sepharose-4B agarose beads (Sigma 4B-200) were activated with cyanogen bromide as described

in section 2.3.2. Canton S CHAPS solubilized microsomal proteins (12 mg), dialized overnight at 4°C against coupling buffer, were attached to the activated Sepharose as previously described (section 2.3.2). A short (5 x 1 cm) column was made from this Canton S coupled Sepharose-4B and washed with PBS-Tween (.05% Tween 20 in PBS). Immune serum from the primary bleed was loaded onto the column and eluted off with PBS-Tween. Serum containing fractions, as indicated by their brown colour (contaminating hemoglobin) were pooled. These Canton S blocked fractions contained strain IIID specific antibody (Abl-B).

2.5.4 Characterization of the Antibody

To determine the specificity of the antibody against cytochrome P450 in Drosophila, the purified IgG fraction was used to precipitate out cytochrome P450 from CHAPS solubilized microsomes. CHAPS solubilized microsomes (40 µl, equivilant to .3 nmoles cytochrome P450) from the resistant strain IIID were incubated at room temperature for 0, 1 or 16 hours in .5 ml 10 mM KPO_4 pH 8.0 + .1% CHAPS and an equal volume of spectral buffer (see 2.3.3) with or without 7 mg Ab2-IgG. After incubation all samples were centrifuged 10 minutes in a microfuge (10,000xg) and the resulting supernatants were assayed for cytochrome P450 content using CO difference spectra. Pelleted material was washed once with PBS (.15 M NaCl in .01 M $NaPO_4$ pH 7.2), resuspended in 20 µl PBS and 20 µl 2x sample buffer, boiled 3 minutes and

electrophoresed on 10% SDS polyacrylamide gels. Gels were subsequently silver stained or Western blotted and probed with Ab2-IgG as the primary antibody (described in section 2.5.5) to examine polypeptides precipitated by the antibody.

2.5.5 Western Immunostaining

Primary antibody (Ab1-B or Ab2-IgG), diluted 1/1000 in PBS-Tween, was applied to blocked blotted nitrocellulose for 2 hours at room temperature with gentle agitation. Excess antibody was washed off with three changes of PBS-Tween for 5 minutes each. Filters were then incubated with secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Inter Medico, TAGO immunologicals 6530)), diluted 1/5000 in PBS-Tween. Incubation and washing conditions were as described for the primary antibody. Alkaline phosphatase was developed using the nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) system (Blake et al, 1984). Alkaline phosphatase substrate (.02 ml 2 M $MgCl_2$, 1 ml 1 mg/ml NBT (Sigma N6876) in 0.1 M Tris-HCl pH 9.2, 0.1 ml 5 mg/ml BCIP (Sigma B8503) in N,N-dimethyl formamide, 9 ml 0.1 M Tris-HCl pH 9.2) was added and the filter was incubated at 37^{o}C until purple bands developed. A brief wash in PBS-Tween removed traces of substrate. The stained filter was stored after air drying. For detection of β-galactosidase, anti-β-galactosidase mouse monoclonal antibody (Promega Z3781, a gift of Dr. W.E. Rawls, McMaster Medical Center)

was used as the primary antibody at a 1/5000 dilution and was detected with goat anti-mouse IgG (H + L) alkaline phosphatase conjugate obtained from Biorad (172-1015) at a 1/5000 working dilution (a gift of Dr. S.T. Bayley, Department of Biology, McMaster University).

Western blots prepared from rabbit IgG-precipitated strain IIID microsomal proteins (section 2.5.3) had to be treated slightly differently. Because the material applied to the gel also contained rabbit IgG (which could react with the secondary goat anti-rabbit antibody during Western immunostaining possibly confusing the results) the rabbit IgG was first blocked by incubating the filter with horse radish peroxidase (hrp) conjugated goat anti-rabbit IgG antibody (Biorad 170-6515, a gift of Dr. A. Oaks, Department of Biology, McMaster University), at a 1/500 dilution for two hours at room temperature. After washing, the filter was reacted with primary and secondary antibody in a normal fashion as described above and stained using the alkaline phosphatase substrate. After photographing the stained blot, the hrp conjugated antibody was overstained using hrp substrate (.01% H_2O_2 in 1 vol 3 mg/ml 4-chloro-1-napthol (Sigma C8890) in methanol; 5 vol 50 mM Tris pH 7.4, 200 mM NaCl) at room temperature until purple bands developed (Hawks, 1982). Colour development was halted by washing with cold water.

2.6 Bacterial Culture Techniques

2.6.1 Bacterial Strains Used

E. coli C600:hfl150, (F^- thi-1 thr-1 leuB6 lacY1 tonA21 supE44 λ^-hflA150 (chr::Tn10)), used for growing lambda gt10 phage was supplied with the lambda gt10 library by Dr. T. Kornberg (Department of Biochemistry and Biophysics, University of California, San Francisco). *E. coli* Y1088, Y1089, and Y1090 were all provided as BACTi DISKS^{TM} in the Lambda gt11 Cloning System kit from Bethusda Research Laboratories (BRL 5272SA). They were reconstituted as recommended by the manufacturer. Their genotypes are; Y1088: ΔlacU169, supE, supF, $hsdR^-$, $hsdM^+$, metB, trpR, tonA21, proC::Tn5 (pMC9); Y1089: ΔlacU169, $proA^+$, Δlon, araD139, strA, hflA[chr::Tn10] (pMC9); Y1090: ΔlacU169, $proA^+$, Δlon, araD139, strA, supF[trpC22::Tn10] (pMC9) (pMC9 = pBR322-$lacI^Q$). *E. coli* MV1190 (Δ(lac-proAB), thi, supE44, Δ(srl-recA), 306::Tn10(tet^r); F': traD36, proAB, $lacI^qZ\Delta m15$), used for pUC119 transformations was a gift of Dr. S. Mak (Department of Biology, McMaster University).

2.6.2 Preparation of Plating Cells

All plating cells were freshly prepared in the same manner (Huynh *et al*, 1986). A 5 ml culture, inoculated from an overnight culture was grown to OD_{600} = 1.0 at 37°C in Luria-Bertani broth (LB; 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl in 1 l ddH_2O, pH 7.5, Maniatis *et al*, 1982) + .2% maltose with or

without 50 µg/ml ampicillin (Sigma A9518). Cells were pelleted in a bench top centrifuge and resuspended in 1/2 volume 10 mM $MgSO_4$ and stored on ice until needed.

2.6.3 Preparation of Competent MV1190 Cells

MV1190 cells were made competent through a modification of the $CaCl_2$ method of Mandel and Higa (1970). A 50 ml culture in 2x Yeast Tryptone broth (16 g Bacto-tryptone, 10 g Bacto-yeast extract, 5 g NaCl, 2.3 g K_2HPO_4 in 1 l ddH_2O, pH 7.5, Miller, 1972) was inoculated with .5 ml of an overnight MV1190 culture and grown at 37°C to OD_{600} of .2. Cells were quickly pelleted (6000 rpm, 1 min), resuspended in 25 ml cold .1 M $MgCl_2$ and quickly pelleted again. After resuspending in 2 ml cold .1 M $CaCl_2$ they were incubated on ice for at least one hour before use.

2.6.4 Transformation of Competent MV1190 Cells

Competent MV1190 cells were transformed with plasmid DNA carrying ampicillin resistance markers for selection, using a method adapted from Mandel and Higa (1970). Briefly 100 µl competent cells and 5 µl (approximately 40 ng) ligated plasmid DNA were mixed on ice for 30 minutes in 12 x 75 mm tubes (Falcon 2063), heat shocked for 2 minutes at 42°C and incubated on ice for another 30 minutes. After warming to room temperature, 1 ml LB broth was added, and the cells were grown at 37°C with shaking for one hour. Fifteen minutes prior to the end of the hour, 20 µl

ampicillin (25 mg/ml in ddH_2O) was added to reduce the number of nontransformed background cells. Aliquots (150 µl) were plated out on LB indicator plates (1.5% agar in LB containing 300 µg/ml ampicillin, 1 mM isopropylthio-β-D-galactoside (IPTG, BRL 5529UA), and 50 µg/ml 5-bromo-4-chloro-3-indolyl-β-D- galactoside (X-gal, BRL 5520UB)). Transformants containing plasmids with DNA inserted into the β-galactosidase gene appeared as white colonies; those without inserts were blue.

2.6.5 Analysis of Recombinant Plasmids from MV1190 Transformants

Miniplasmid preparations were made from recombinant transformants using the "boiling method" (Holmes and Quigley, 1981) and analysed by restriction endonuclease digestion followed by agarose gel electrophoresis. A 5 ml overnight culture of transformed MV1190 cells in LB containing 50 µg/ml ampicillin was pelleted in a bench top centrifuge. Cells were resuspended in 350 µl STET buffer (8% sucrose, 5% Triton, 50 mM EDTA, 50 mM Tris pH 8.0) and transferred to a 1.5 ml eppendorf tube. Lysozyme (25 µl of 20 mg/ml in 10 mM Tris pH 8.0, Sigma L6876) was added and the tube was immediately placed in a boiling water bath for 45 seconds. Cellular DNA and debris were pelleted for 10 minutes in a microfuge and the pellet was removed. The remaining fluid was subjected to one phenol/chloroform (1:1) extraction. Nucleic acids were precipitated with the addition of one volume isopropanol at room temperature for 30 minutes. DNA was pelleted

for 10 minutes, washed with 70% ethanol and air dried. The pellet was resuspended in 50 µl TE (10 mM Tris pH 7.5, 1 mM EDTA). Restriction analysis was carried out on 5 µl samples. Clones containing inserts of interest were digested on a large scale, isolated on and excised from a gel, purified and oligolabelled for use in probing Southerns and Northerns.

2.7 DNA Analysis

2.7.1 Nucleic Acid Quantitation

DNA and RNA were quantified by examining the UV spectrum of suitably diluted samples between 200 and 300 nm. The absorbance at 260 nm was used to calculate nucleic acid concentration assuming that 1 OD unit is equivalant to 50 µg/ml dsDNA or 40 µg/ml RNA (Maniatis et al, 1982). The ratio of OD_{260}/OD_{280} was used to assess the purity of the sample. Pure DNA preparations have a ratio of 1.8, while pure RNA preparations have a ratio of 2.0.

2.7.2 Restriction Endonuclease Digestion

DNA was restricted in a small volume (20 µl) with the appropriate buffer conditions and temperature as recommended by the enzyme manufacturer. Restriction enzymes were obtained from BRL. One unit of enzyme was used to digest 1 µg of DNA. Samples were analysed by agarose gel electrophoresis.

2.7.3 Agarose Gel Electrophoresis

DNA separations were made on agarose gels using SeaKem HEEO agarose (Mandel 50031) in a 1x TBE buffer (90 mM Tris, 2.5 mM EDTA, 90 mM Boric acid). Agarose was dissolved in 1x buffer with heating and allowed to cool to approximately 55^{o}C before pouring into the gel tray. After solidifying 1x TBE buffer was added to a depth of 1-2 mm above the gel. Samples were mixed with 1/10 volume agarose gel loading dye (50% glycerol, 10 mM EDTA, 1% SDS, 0.1% BPB), heated to 65^{o}C 10 minutes and cooled on ice before loading. The gels were electrophoresed at 5 V/cm. HindIII digested lambda DNA was used as a molecular weight marker. Gels were stained with .5 µg/ml ethidium bromide in buffer or water and destained in water. DNA was visualized with a short wave UV illuminator (Fotodyne, Inc) and was photographed using a Polaroid MP4 land camera, Polaroid 545 land film holder and a Toshiba R60 filter and Polaroid type 57 land film.

2.7.4 Oligolabelling

DNA probes were made radioactive by using the oligolabelling system from Pharmacia. DNA (50 ng) was heat denatured at 90^{o}C for 15 minutes and cooled to 37^{o}C for 5 minutes. Following the protocol described by Pharmacia, primer extension from random hexanucleotide oligomers incorporated dCTP-α-^{32}P (ICN R33004X) into the probe DNA. After termination of the reaction, unincorporated nucleotides were removed using the spin column

technique (Maniatis et al, 1982). Sample radioactivity was measured using a Beckman LS 1801 counter before and after removing free nucleotides, to determine the percent incorporation of label into the DNA molecule. Prior to using the probes in hybridizations, the DNA was denatured by adding 1/10 volume 1 N NaOH, heating at 37^{o}C for 5 minutes and neutralizing with 1/10 volume 1 N HCl (Wahl and Berger, 1987).

2.7.5 Endlabelling of Lambda HindIII DNA

Lambda HindIII digestions were end labelled to serve as radioactive molecular weight markers for Southern gels (Drouin, 1980). Isotope was incorporated into the ends of the HindIII fragments by incubating .2 µg digested DNA with 10 uCi dATP-α-^{32}P (ICN R33002X) and 1 unit of Klenow. After a 20 minute room temperature incubation the reaction was halted with 1 µl .5 M EDTA and diluted 10 fold with water.

2.8 Lambda gt11 Preparation

A lambda gt11 library was prepared by isolating cDNA inserts from a lambda gt10 library and ligating them into lambda gt11 arms. The Drosophila cDNA lambda gt10 library was a gift of Dr. T. Kornberg (Department of Biochemistry and Biophysics, University of California, San Francisco).

2.8.1 Preparation of Lambda gt10 Phage

Lambda gt10 phage were prepared as described by Davis et al (1980). Phage from larval and male and female adult libraries were plated out on 150 mm Petrie plates on an E. coli hfl host in the following manner. Phage ($4x10^4$) were adsorbed to .2 ml E. coli C600:hfl plating cells for 15 minutes at 37°C. LB soft agar (7 ml of .7% Bacto agar in LB) was added to the cells, mixed and poured over a 150 mm LB petrie plate (1.5% Bacto agar in LB) containing .2% glucose. Plates were incubated right side up at 37°C for 6 hours, transferred to 4°C and overlaid with 10 ml lambda diluent (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA) overnight, allowing the phage to diffuse into the overlay. The overlay was recovered from the plates and centrifuged at 5000 rpm to remove any bacterial debris. Phage were pelleted in a Beckman SW27 rotor at 23,000 rpm at 4°C for 90 minutes, and resuspended in one ml lambda diluent. Two CsCl block gradients were run to remove nucleic acids and proteins (Davis et al, 1980). CsCl cushions were made in 5 ml pollyallomer ultracentrifuge tubes (Beckman 326819). A step down gradient was 1 ml 5 M CsCl in gradient buffer (10 mM $MgSO_4$, 10 mM Tris pH 8.0, 0.1 mM Na_2EDTA) layered under 3 ml 3 M CsCl. Phage (1 ml) in lambda diluent were layered on top of the CsCl cushions. After centrifuging 1 hour at 30,000 rpm in an SW50.1 rotor 20°C, the resulting bluish band of phage at the interface of the 3 M and 5 M

solutions was removed (up to .5 ml) with a 25g needle attached to a 1 ml syringe. This was mixed with an equal volume of 7.2 M CsCl in gradient buffer and layered under 3 ml 5 M and 1 ml 3 M CsCl. Following centrifugation the phage at the interface of the 3 and 5 M solutions were removed. This purified phage preparation was used to prepare phage DNA.

2.8.2 Preparation of Lambda gt10 DNA

DNA was prepared from the isolated phage using formamide (Davis et al, 1980). Phage isolated from above were mixed in a 15 ml Corex centrifuge tube with 1/10 volume 2 M Tris pH 8.5, 0.2 M Na_2EDTA and one volume deionized formamide, and incubated at room temperature for 30-60 minutes. One volume of water (to prevent precipitation of CsCl) and six volumes of absolute ethanol were added, the preparation was gently mixed and DNA was quickly pelleted in a Sorvall RC-2 centrifuge using an SS34 rotor, by increasing the speed to 10,000 rpm, then immediately allowing it to return to zero. This quick spin pelleted the DNA but not the empty phage particles. Pellets were washed several times in 70% Etoh and allowed to air dry before being resuspended in 100 µl TE buffer. Heating to 65°C for 5-10 minutes aided in dissolving the DNA by separating the sticky ends at the cos sites of lambda. To improve restriction endonuclease digestion, the DNA was dialysed overnight against TE, or phenol and chloroform extracted, followed by ethanol precipitation.

2.8.3 Digestion of Lambda gt10 DNA and isolation of inserts

Lambda gt10 DNA was digested with EcoR1 (BRL) to remove insert cDNA using buffer conditions recommended by the manufacturer. After digestion the DNA was separated by .7% agarose gel electrophoresis as described earlier. A portion of the gel containing inserts corresponding in size to 0-7.5 kb was cut out and DNA purified from it using GENECLEAN (BIO 101, Inc.). Because the amount of insert DNA isolated was so small, its concentration was determined using ethidium bromide and a fluorimetric assay. Briefly, plasmid DNA of known concentration was used to prepare a standard curve measuring between 0 and .5 µg DNA. Ethidium bromide (.1 µg) was added to each sample and the amount of fluorescence of each was determined by exciting at 260 nm and measuring emitted light filtered through a 600 nm cutoff filter. Ethidium bromide blanks were used to zero the instrument. Samples of insert DNA were treated identically and the amount of DNA contained in them was calculated from the standard curve. Some contamination of samples with fluorescent particles from the GENECLEANed agarose slices probably occurred, hence the DNA content was probably overestimated.

2.8.4 Ligation and packaging of insert DNA into lambda gt11

Insert DNA (200 ng) from the lambda gt10 library (see 2.8.3) was ligated into 1 µg of dephosphorylated lambda gt11 arms (BRL 5272SA) as described by Huynh et al (1986). Insert and

vector DNA were mixed and precipitated in the presence of .2 M sodium acetate and two volumes cold absolute ethanol by cooling to $-70^{o}C$ for 30 minutes. DNA was pelleted 15 minutes in a microfuge. The resulting small white pellet was washed with 70% ethanol, repelleted for 10 minutes, and resuspended in 4 µl 10 mM Tris pH 7.5, 10 mM $MgCl_2$ + 0.5 µl 10 mM ATP + 0.5 µl 0.1 M DTT. T4 DNA ligase (BRL 5224SB, 0.1 µl of 1.6 mg/ml) was added and the reaction was allowed to proceed at $12-15^{o}C$ overnight. Ligated lambda DNA was packaged using the Biorad *in vitro* packaging kit (170-3460), following the directions of the manufacturer. The kit contained sonic and freeze thaw extracts from lambda prophages defective in different aspects of the packaging reaction. Ligated lambda gtl1 DNA (5 µl) was added to a tube of frozen sonic extracts. After the freeze/thaw extracts had thawed on ice, 15 ul were added to the DNA/sonic extract and gently mixed. The tube was allowed to sit at room temperature for two hours after which .5 ml SM media (5.8 g NaCl, 2 g $MgSO_4 7H_2O$, 50 ml 1M Tris-Cl pH 7.5, 5 ml 2% gelatin in 1 l ddH_2O, Maniatis *et al*, 1982) was added along with a drop of chloroform. Debris was removed by centrifuging 30 seconds in a microfuge. The packaged DNA was now ready to be screened.

2.9 Analysis of the Lambda gtll Library

2.9.1 Screening of the Lambda gtll Library

The percentage of recombinants contained in the lambda gtll library was determined by plating out a small number of phage (100-200) in the presence of 10 mM IPTG and 1 mM X-gal (Huynh et al, 1986). Lambda gtll (50 µl of a 10^{-2} dilution) was mixed with .2 ml Y1088 plating cells and poured with 3 ml LB soft agar containing 40 µl 1 M IPTG and 40 µl 40 mg/ml X-gal onto an 85 mm LB plate and grown overnight at 37°C. Under these conditions nonrecombinant phage (not insert containing) form blue plaques while recombinant phage (insert containing) form clear plaques due to their inability to act on the substrate (X-gal) with their interrupted β-galactosidase gene product. The percentage of recombinants was equivalant to the fraction of clear plaques.

The lambda gtll library was screened on large 150 mm LB plates (Huynh et al, 1986 and Snyder et al, 1987) without further amplification. Approximately $1x10^4$ phage were initially adsorbed to .2 ml Y1088 plating cells for 15 minutes at 37°C, allowing the phage to enter a modification$^+$ / restriction$^-$ host. Y1090 plating cells (.5 ml) were added and the entire mixture was poured with 7 ml top agar onto a large LB plate to allow fusion protein production. The plates were incubated at 42°C for 3 hours then quickly transferred to a 37°C incubator and overlayed with dry nitrocellulose filters previously saturated with 10 mM IPTG.

Incubation continued for a furthur 8-10 hours after which the filters were stained for antigen according to Snyder et al (1987). The positions of the filters were marked on the plates using waterproof ink and a needle. With the exception of TBS all solutions used in staining were freshly prepared. Filters were removed, washed briefly with TBS (150 mM NaCl in 50 mM Tris-HCl pH 8.1) and blocked for 30 minutes at room temperature by shaking with .5% BSA, .05% Tween-20 in TBS. Filters were incubated with primary antibody, Ab2-IgG, diluted 1/500 in TBS + .1% BSA + .05% Tween-20, for two hours at room temperature. After washing the filters with TBS + .1% BSA, TBS + .1% BSA + .1% NP40, and TBS + .1% BSA, for ten minutes each on a rotary shaker, secondary antibody (alkaline phosphatase conjugated goat anti-rabbit IgG antibody), diluted 1/5000 in TBS + .1% BSA + .05% Tween-20 was added for two hours at room temperature. Following a second series of washes, alkaline phosphatase substrate, (section 2.5.5) was added and the filters were incubated for up to 5 minutes at $37^{o}C$. Plaques corresponding to potentially positive clones were picked from the agar plate using the large end of a Pasteur pipette (to ensure the plaque of interest was picked) and added to one ml of SM media. Each potentially positive phage clone was replated and rescreened until pure.

2.9.2 Preparation of Lysogens

Lysogens were prepared from lambda gt11 antigenic clones so that the fusion proteins could be examined in detail. The method was adapted from Dr. B. Cochrane (Department of Biology, University of South Florida, personal communication). E. coli Y1089 was grown to OD_{600} = .5 in LB + tetracycline (Sigma T3383, 25 µg/ml) media and infected with phage at a 10:1 phage to cell ratio. The phage/cell mix was incubated for 15 minutes at room temperature, suitably diluted and plated out on LB + tet plates (100-200 cells per plate). Colonies that grew up overnight at 30°C were picked and replated onto a second LB + tet plate and also a lawn of Y1090 cells (.2 ml Y1090 plating cells poured with 3 ml soft agar onto an LB plate). The LB + tet plate was incubated at 30°C, the Y1090 plate at 42°C. At 42°C, lysogens would be induced and the released phage would infect the surrounding susceptible cells forming a plaque. Colonies that formed plaques at 42°C were considered to be lysogens. The corresponding colony on the 30°C plate was used to prepare a stock culture.

2.9.3 Lysate Preparation

Lysogen lysates were prepared according to protocols established by Huynh et al (1986), Kaufman et al (1986) and Young and Davis (1983a). Freshly plated lysogens were inoculated into a 50 ml liquid culture of LB + tet and grown to OD_{600} = .5 at 30°C

at which point phage induction was initiated by raising the culture temperature to 42°C for 15 minutes. IPTG was added to a final concentration of 10 mM to induce fusion protein production from the lacZ promotor. The cultures were transferred to a 37°C incubator with shaking until the cells started to lyse (1-2 hours, as determined for each lysogen). The cells were harvested at room temperature (10 min, 2500 rpm) and resuspended in 1/50 volume TBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma P7626) to inhibit proteolytic degredation. The resuspended cells were aliquoted, frozen in liquid nitrogen and stored until needed at -70°C. Upon thawing the cells were essentially lysed. It was important not to allow the thawed cells to sit at room temperature for longer than necessary as this increased their viscosity due to a release of genomic DNA. If samples did become viscous however, sonication (six 1 sec pulses at output 3 on a Sonifer cell disruptor), aided in reducing viscosity by shearing this genomic DNA. Sonication was also useful in lysing remaining cells. After thawing, debris was pelleted for 10 minutes at 4°C in a microfuge and protein in the supernatant was analysed by 5% SDS polyacrylamide gel electrophoresis as described in section 2.4.2, followed by silver staining and Western immunoblotting (section 2.4.4) and immunoanalysis (section 2.5.5).

2.9.4 Preparation of Lambda gtll DNA

A high titer phage lysate was prepared from lambda gtll clones as described by Davis et al (1986). This high titer lysate was used in lysogen formation and to prepare lambda gtll phage DNA. DNA was prepared exactly as described by Davis et al (1986). After the final ethanol precipitation it was necessary to remove RNA before analysis. This was accomplished by adding RNase (BM 109 142, 10 µg/ml) to the nucleic acids, incubating at room temperature for 30 minutes, then phenol and chloroform extracting and ethanol precipitating. The final DNA pellet was resuspended in 100 µl TE and the DNA content was determined.

2.9.5 Cloning Inserts into pUC119

Lambda gtll inserts from clones of interest were excised from lambda gtll using appropriate restriction enzymes and ligated into pUC119 (Vieira and Messing, 1987) at the multicloning site using the ligation procedure described earlier. The ligated DNA was diluted with 20 µl TE and 5 µl aliquots were transformed into competent MV1190 cells and analysed.

2.10 Analysis of Genomic DNA and RNA

2.10.1 Genomic DNA Preparation

Drosophila genomic DNA was prepared according to Bender et al (1983) with a few modifications. Tissue (100 mg whole flies) was homogenized in 2 ml of fly DNA buffer (100 mM NaCl, 200 mM

sucrose, 100 mM Tris-HCl pH 9.1, 50 mM EDTA, 0.5% SDS) and incubated at 65°C for 30 minutes. Potassium acetate (.3 ml 8 M) was added and the homogenate incubated on ice for 30 minutes. After pelleting fly debris (10,000xg, 5 min) nucleic acids were precipitated from the supernatant with an equal volume of absolute ethanol for 5 minutes at room temperature. Nucleic acids were pelleted at 10,000xg in an SS34 rotor for 5 minutes, washed with 70% ethanol, dried and resuspended in 100 µl TE buffer. Insoluble material was pelleted out and the resulting solution was subjected to an RNase (10 µg/ml) treatment followed by phenol, phenol/chloroform and chlorofom extractions. A second ethanol precipitation followed and the resulting DNA was resuspended in 100 µl TE.

2.10.2 DNA dot blots

Genomic DNA from Canton S and IIID strains was dot blotted onto nitrocellulose (Schleicher and Schuell, BA85) according to protocols established by the membrane manufacturer (Schleicher and Schuell, 1987). Genomic DNA (30 µg) was denatured with 0.1 vol 3 M NaOH at 60°C for 1 hour. After cooling to room temperature 10x SSC (.3 M sodium citrate, 3 M NaCl pH 7.0) was added to a final concentration of 6x. DNA was applied in 2 µl aliquots to a nitrocellulose membrane previously saturated with 6x SSC. Each 2 µl application was allowed to airdry before subesequent ones were made on top. Plasmid DNA samples (200 ng) from which probe

fragments were isolated, were treated identically and served as positive controls for the probe used.

2.10.3 Southern Blotting

DNA, separated on agarose gels, was transferred to nitrocellulose (S&S BA85) using the Southern technique (Southern, 1975) as described by Maniatis et al (1982). After staining with ethidium bromide and photographing, gels were denatured, neutralized and transferred by capillary action to nitrocellulose using 10x SSC as the transfer buffer. After transfer, filters were baked for two hours at 80°C and stored dry until hybridized.

2.10.4 DNA Hybridizations

DNA hybridizations were carried out as described by Maniatis et al (1982). Baked nitrocellulose filters were prehybridized 2-4 hours at 68°C in .2 ml/cm^2 prehybridization mix (6x SSC, .5% SDS, 5x Denhardt's (.1% Ficoll, .1% polyvinylpyrrolidone, .1% bovine serum albumin (Pentax fraction 5) in ddH_2O), 100 µg/ml heat denatured salmon sperm DNA) in a heat sealable plastic bag. After incubation a corner was cut off and as much as possible of the prehybridization mix was squeezed out. Hybridization fluid (6x SSC, .01 M EDTA, 5x Denhardts, .5% SDS, 100 µg/ml ssDNA) was added (50 µl/cm^2 filter) and oligolabelled, denatured probe was added directly to the bag. The bag was heat sealed, contents were mixed and hybridization was continued 16-24

hours at 68°C. After hybridization filters were removed from the bag and washed 5 minutes at room temperature in 2x SSC, .5% SDS, followed by a 15 minute wash at room temperature in 2x SSC, .1% SDS. A high stringency wash at 68°C for 2 hours in 0.1x SSC, .5% SDS was repeated with fresh solution for 30 minutes. After air drying, the filter was exposed to Kodak XAR5 film at -70°C with an intensifying screen for 1-3 days, and then developed. Low stringency hybridizations were carried out at 42°C.

2.10.5 RNA Preparation

Total RNA was prepared from larval, pupal and adult stages of Canton S and IIID strains using the hot phenol/chloroform method exactly as described by Jowett (1986). RNA was resuspended in water and quantified as described earlier (section 2.7.1). Precautions were taken to avoid RNase contamination. All solutions were prepared with 0.1% diethyl pyrocarbonate (DEPC, Sigma D5758) treated water and autoclaved. Non sterile plasticware was washed with 0.1% DEPC in water prior to autoclaving and glassware was baked at 250°C for 4 hours. Gloves were worn at all times.

2.10.6 RNA dot blot

RNA samples were dot blotted onto S&S nitrocellulose (BA85) as described by the membrane manufacturer (Schleicher and Schuell, 1987). RNA was denatured by adding .6 volume 20x SSC and

.4 volume 37% formaldehyde and incubating at 60°C for 15 minutes. RNA was applied in 2 µl aliquots to 15x SSC saturated nitrocellulose membrane, allowing the membrane to dry between applications. In order to compare relative intensities of hybridization between strains and with various probes, RNA was applied to the filter in a dilution series, loading between .5 and 5 µg. Denatured plasmid DNA (section 2.10.2) was also dotted on to serve as positive controls for the probe. All filters were baked at 80°C for 30 minutes prior to hybridization.

2.10.7 RNA Hybridizations

RNA dot blots were hybridized with oligolabelled, denatured DNA probes as described by the membrane manufacturer (Schleicher and Schuell, 1987). Prehybridization fluid (50% deionized formamide, 5x Denhardt's, 0.5% SDS, 5x SSPE, 100 µg/ml ssDNA) was added to the filter in a heat sealable plastic bag and incubated at 42°C for 30 minutes. 20x SSPE is 3.6 M NaCl, 200 mM NaH_2PO_4 pH 7.4, 20 mM EDTA in ddH_2O. Hybridization was carried out in the same buffer with oligolabelled, denatured DNA probes at 42°C 12-18 hours. After hybridization the blot was washed at room temperature in 2x SSPE, 0.1% SDS (2x 10 min) and in 0.1x SSPE, 0.1% SDS (2x 10 min). A stringent wash followed in 0.1x SSPE, 0.1% SDS at 42°C for 1 hour, after which the filter was air dried and exposed to Kodak XAR5 film at -70°C with intensifying screens. In addition to probing with the probes of interest, all RNA dot

blots were also probed with a 400 bp Drosophila 5C actin insert from the plasmid pActin (a gift of Dr. W. McGinnis, Department of Molecular Biophysics and Biochemistry, Yale University). This served as an internal control for the amount of actin mRNA present as this should not vary between strains or stages in the Drosophila life cycle.

RESULTS

3.1 General Analysis of Canton S and IIID Strains

The malathion resistant strain IIID, used in the preparation of cytochrome-P450-directed antibodies, originated from a locally caught (Hamilton, Ontario) population of flies. A mixture of isofemale lines, made from this population, were selected by exposure to the insecticide malathion to create a resistant population (Singh and Morton, 1981). After 110 generations at high malathion selection pressure, isochromosomal strains were made for each of the three major chromosomes (Halpern and Morton, 1987). One of these, strain IIID, homozygous for a resistant third chromosome is the basis of this study. It is compared to Canton S, a susceptible wildtype laboratory strain, in Table 1. The actual resistance of the two strains was measured by determining the LC50 (the concentration of malathion required to kill 50% of adults over a 24 hour period) of each. Strain IIID was able to survive exposure to malathion at levels more than ten times higher than that of Canton S. This increased resistance is due in part to increased mixed function oxidase (mfo) activity, an enzyme system which is involved in the detoxification of compounds entering the body. Using a 7-ethoxycoumarin assay to measure mfo activity, it was shown that strain IIID had increased levels of activity when compared to Canton S. This four fold increase in

Table 1: Characteristics of D. melanogaster strains Canton S and IIID

	Cytochrome P450 nmole/mg protein	MFO activity pmole/sec/mg protein	LC50 uM	AchE activity nkals/mg protein
CS	0.18 (.007)	0.3 (0.1)	6 (2)	16.9 (1.3)
IIID	1.07 (.14)	1.2 (0.2)	80 (8)	7.5 (0.5)

mfo activity correlated with a five fold increase in cytochrome P450, an enzyme component of the mfo system. Increased levels of cytochrome P450 have often been correlated with insecticide resistance. Cytochrome P450 content measurements, made using CHAPS solubilized microsomes, indicated that strain IIID had contents five times higher than that of Canton S. Similar values were obtained when using nonsolubilized microsomal preparations (data not shown). Acetylcholinesterase (AchE) is a target molecule for many organophosphates including malathion. Morton and Singh (1982) have shown a negative correlation between acetylcholinesterase activity and resistance. In addition they have noted variations in the electrophoretic mobility of the molecule in resistance strains. Strain IIID typically had levels of AchE activity only 40% that of Canton S.

3.2 Analysis of Microsomal Proteins

3.2.1 Microsomal Preparation

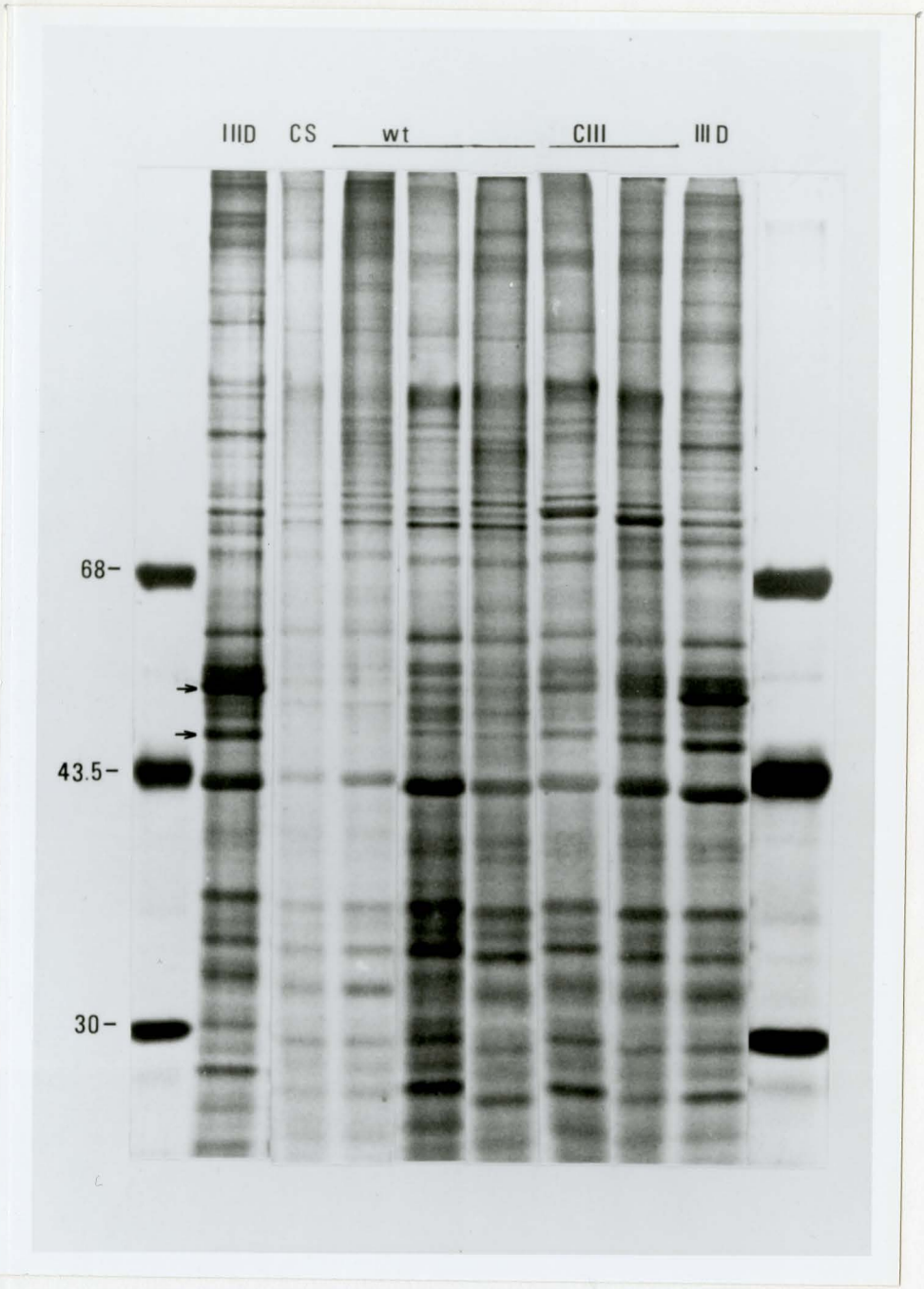
As previously described, strain IIID had significantly increased levels of cytochrome P450, a microsomal membrane protein, when compared to Canton S. To examine this phenomenon, microsomal proteins from strain IIID and the nonresistant strain Canton S were examined with particular attention paid to proteins within the cytochrome P450 molecular weight range of 43 - 60 kd (Nebert et al, 1982). Microsomal membranes, separated from fly homogenates by high speed centrifugation (135,000xg) were heat

denatured and examined for protein with SDS-PAGE (Figure 1). Silver staining indicated that some differences in protein content did exist between the resistant and nonresistant strains. In strain IIID, two polypeptides of 50 and 54 kd were present in enhanced quantities when compared to Canton S. Differences between strain IIID and Canton S appeared to relate only to the amount of protein present, as no new polypeptides were observed in strain IIID. Other nonresistant wildtype strains or strains homozygous for a resistant chromosome III (Halpern and Morton, 1987) were similar in their protein profiles to Canton S or strain IIID respectively. Because the two enhanced proteins found in strain IIID (50 and 54 kd) were within the molecular weight range of cytochrome P450, future efforts focused on them.

3.2.2 Microsomal Solubilization

The microsomal membranes pelleted by high speed centrifugation contained a large number of surface and membrane bound proteins. In order to further purify integral membrane proteins, including cytochrome P450, microsomal membranes were solubilized using detergent. Detergents form micelles around hydrophobic membrane proteins, releasing them from the membranes. Non soluble proteins and membrane fragments could then be removed by high speed centrifugation (135,000xg) leaving the solubilized proteins in the supernatant.

Figure 1: SDS polyacrylamide gel, stained for total protein, of microsomal proteins from various different wildtype (lanes 2 through 5) and resistant chromosome III (lanes 1 and 6 through 8) Drosophila melanogster strains. Canton S and strain IIID lanes are indicated. The arrows indicate the enhanced proteins of 50 and 54 kd observed in strain IIID microsomal samples. Modified from Houpt et al (1988).


IIID
CS
wt
CIII
III D
68-
43.5-
30-

The effect of CHAPS, a zwitterionic detergent, on microsomal cytochrome P450 was compared to sodium cholate, an anionic detergent, by measuring cytochrome P450 content after the addition of detergent. Washed microsomes from the resistant strain IIID were homogenized in phosphate buffer, phosphate buffer plus .4% or .6% sodium cholate or phosphate buffer plus .5% CHAPS, and nonsoluble material was removed from detergent containing samples by ultracentrifugation. The supernatants were assayed for cytochrome P450 content using CO difference spectra (Figure 2). In the absence of detergent some cytochrome P450 was present however the addition of detergent (either sodium cholate or CHAPS) did enhance the amount obtained. Sodium cholate at both concentrations resulted in the production of cytochrome P420, an altered inactive form of cytochrome P450. Of the three detergents used, CHAPS at .5% resulted in the most cytochrome P450 with very little contaminating cytochrome P420. Moldenke et al (1984) have also noted an appreciable increase in cytochrome P450 yield when using CHAPS for solubilizations.

In subsequent microsomal preparations CHAPS was added to a final concentration of .5% (w/v) to resuspended, pelleted microsomes. Nonsoluble proteins were pelleted out and discarded and the supernatants were saved. Figure 3 shows the effect of CHAPS on microsomal purification in strain IIID and Canton S. Microsomes homogenized in buffer only or in buffer plus .5% CHAPS

Figure 2: CO difference spectra of strain IIID microsomal membranes solubilized with (a) no detergent, (b) .4% sodium cholate, (c) .6% sodium cholate or (d) .5% CHAPS.

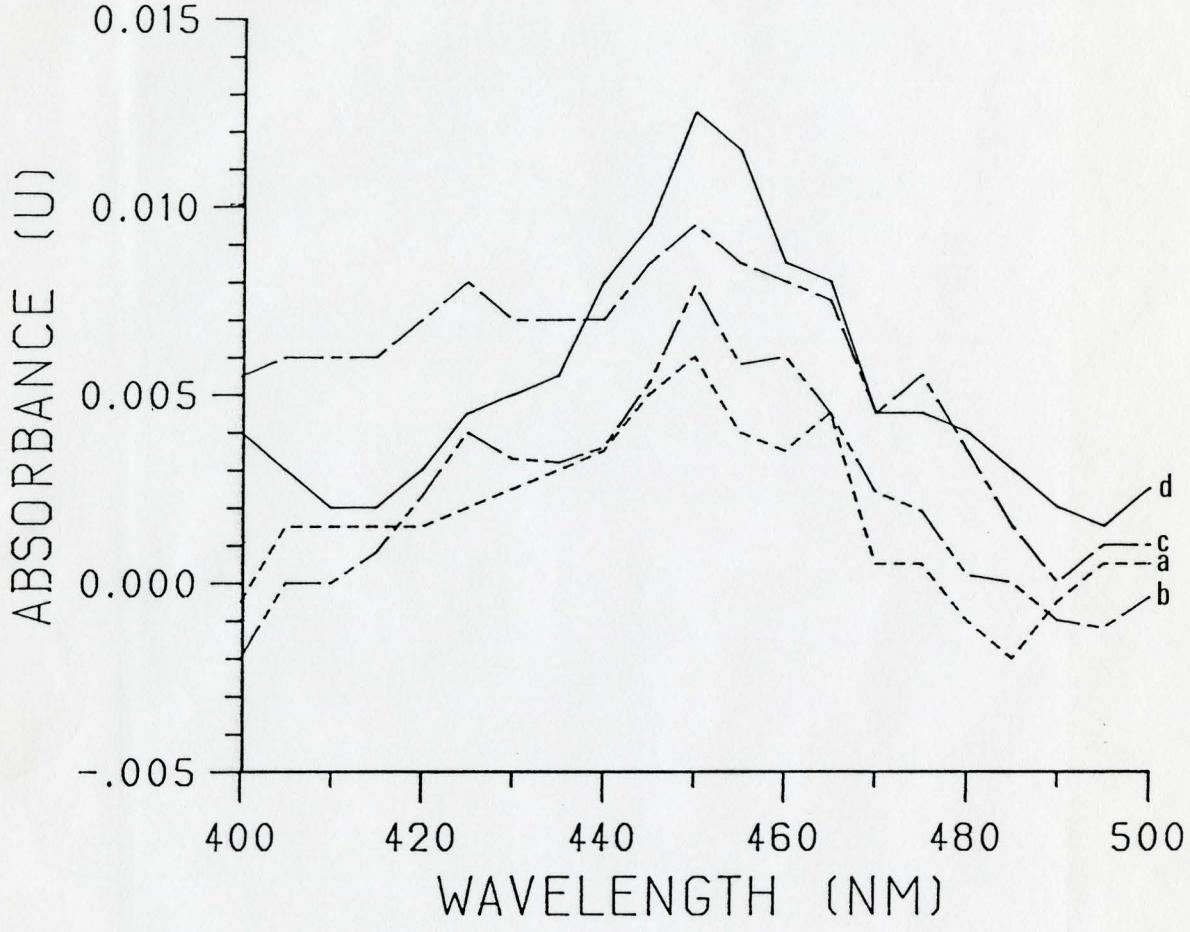
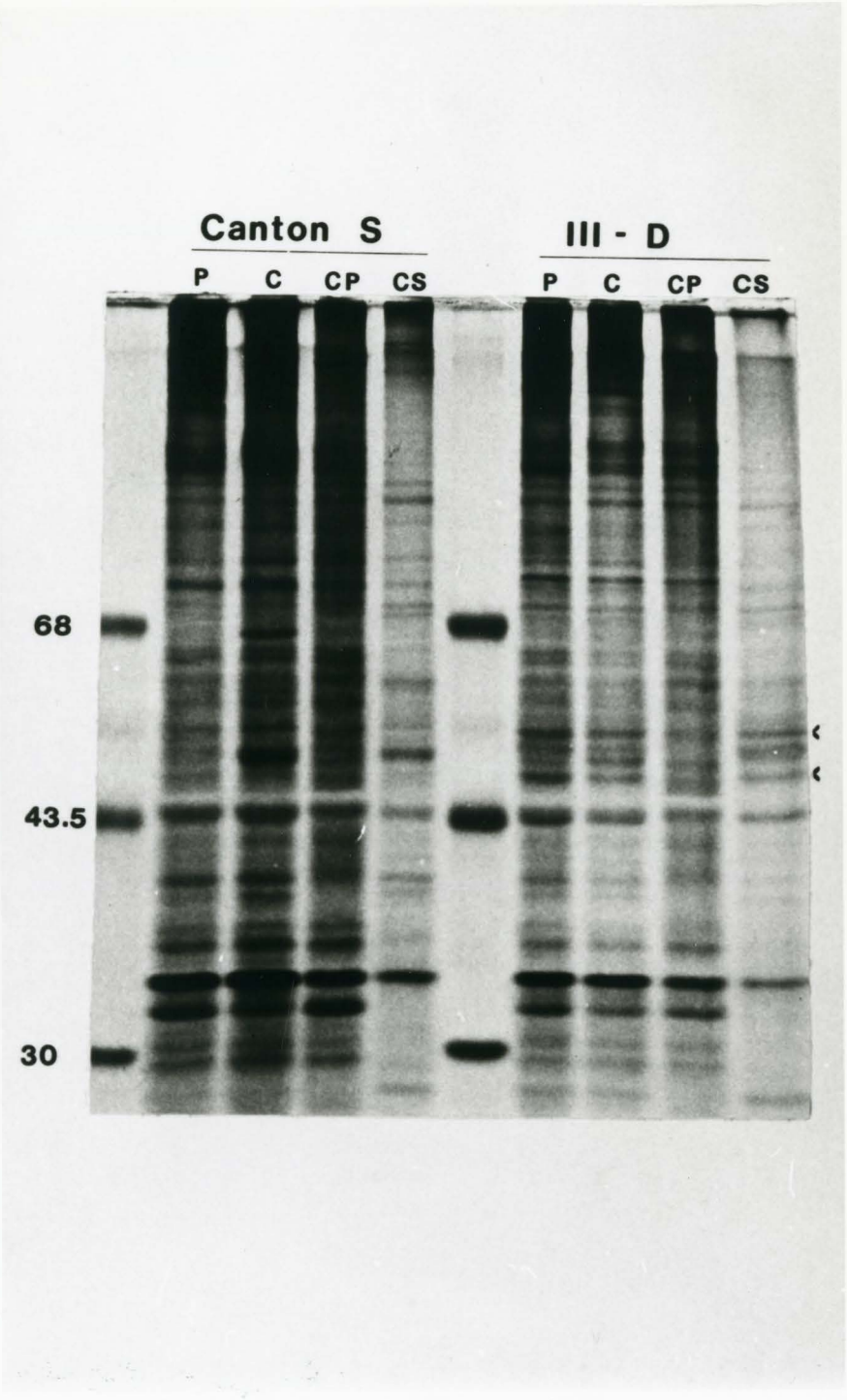

0.015
0.010
0.005
0.000
-.005
ABSORBANCE (U)
400
420
440
460
480
500
WAVELENGTH (NM)
d
c
a
b

Figure 3: Silverstained SDS gel showing the effect of CHAPS on microsome solubilization in Canton S and strain IIID. Microsomal proteins (10 µg) were applied to the gel before solubilization (P) and after the addition of .5% CHAPS (C). Following high speed centrifugation nonsoluble pelleted material (CP) and CHAPS soluble material (CS) were also examined. The "<" indicate the enhanced proteins of 50 and 54 kd observed in strain IIID samples.


Canton S
III - D
P
C
CP
CS
P
C
CP
CS
68
43.5
30

were examined by SDS-PAGE. Also examined was material pelleted or retained in the supernatant after CHAPS solubilization and high speed centrifugation. In both Canton S and strain IIID approximately 40% of the microsomal protein was pelleted as nonsoluble material including most proteins larger than 68 kd. There was an enhancement of proteins in the 45 - 65 kd region in the supernatant of samples solubilized with CHAPS after nonsoluble material was removed. In Canton S one prominent protein (52 kd) was further purified by CHAPS solubilization as were three prominent proteins in strain IIID, including the two enhanced proteins (50 kd and 54 kd) described in Figure 1. Based on CO difference spectra, approximately 80% of the total cytochrome P450 was retained in the CHAPS supernatant while 20% was lost in the CHAPS pellet.

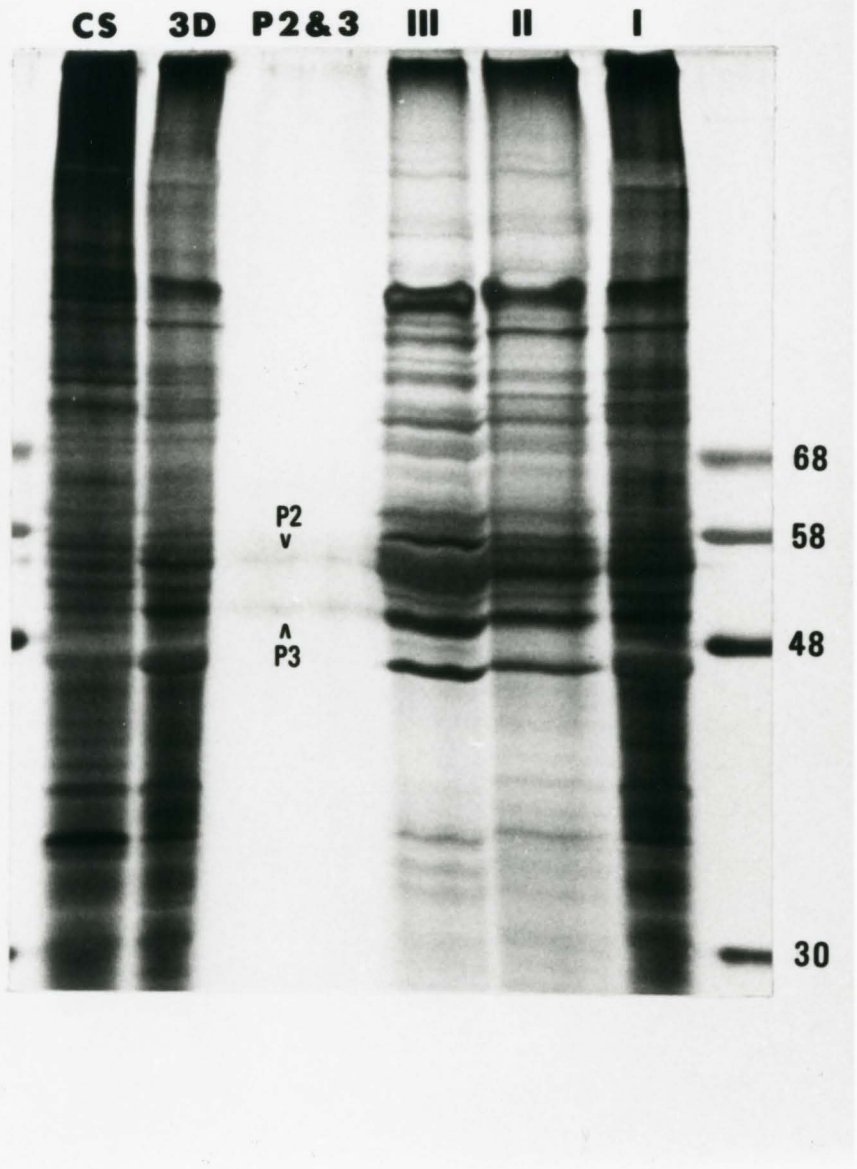
3.2.3 Column Purification of Microsomes

Cytochrome P450 from the resistant strain IIID was further purified from CHAPS solubilized microsomes by DEAE cellulose and hydrophobic affinity chromatography resulting in more than a three fold increase in its relative concentration. The effectivenenss of each step is summarized in Table 2 and by SDS polyacrylamide gel analysis in Figure 4. CHAPS solubilized microsomes (fraction I) were loaded onto a DEAE cellulose column and proteins were eluted off with .3% CHAPS plus .175 M NaCl. Protein containing fractions, as indicated by a brown colour (due to the presence of

Table 2: Purification of IIID Microsomal proteins

	Total Protein mg	Total Cyt P450 nmole	Total Cyt P450/ Total Protein nmole/mg
Fraction I	45.6	28.3	0.6
Fraction II	15.0	17.0	1.1
Fraction III	5.4	11.9	2.2

Figure 4: Silverstained SDS gel showing column purification of strain IIID microsomal proteins. Lanes 1 and 2 contain CHAPS solubilized microsomal proteins from Canton S and strain IIID respectively. Lanes 6, 5 and 4 represent the steps in purification as described in the text. Lane 3 contains gel isolated P2 and P3 (section 3.2.4). Reprinted from Houpt et al (1988).


CS
3D
P2&3
III
II
I
68
58
48
30
P2
v
^
P3

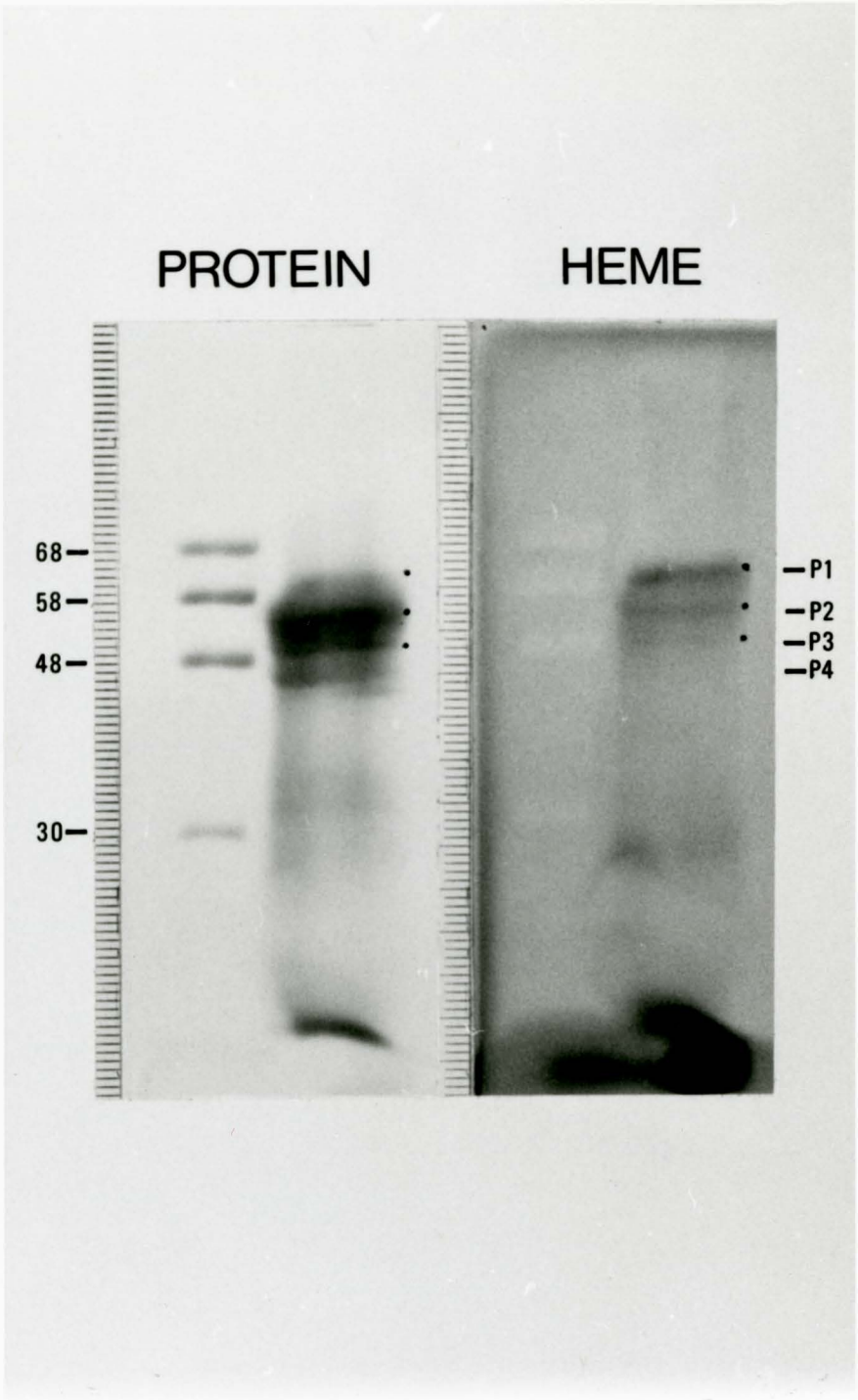
cytochromes) were collected and pooled (Fraction II). This step removed most proteins less than 48 kd in size (Figure 4) without apparently removing much of either of the two strain IIID specific enhanced proteins (50 kd and 54 kd). Although approximately 30% of the total cytochrome P450 was lost in this step, its specific activity (nmoles/mg) was increased almost two fold. Fraction II was then applied to an octylamino-Sepharose-4B hydrophobic column and protein was eluted off with .3% CHAPS in a 0 - .5 M NaCl gradient (fraction III). Although there was little difference in the protein spectrum between the hydrophobic column and the DEAE column (Figure 4) cytochrome P450 concentration was increased another two fold. Carbon monoxide difference spectra on fractions II and III indicated little or no conversion to cytochrome P420 had occurred during the purification procedure. Fraction III was subsequently used in the production of antibodies and examined for the presence of heme-containing proteins.

3.2.4 Heme Staining

Cytochrome P450 is a heme-containing protein in which apo-enzymes combine with porphyrin rings forming holo-enzymes (Nebert et al, 1982). Because the heme group is not covalently bound to the rest of the protein, it is lost during the denaturation processes necessary for SDS-PAGE. Total loss can be prevented and heme can be subsequntly stained by loading large amounts of protein and avoiding denaturing conditions such as heat

and removing β-mercapto ethanol from the sample buffer. Strain IIID CHAPS solubilized, column purified microsomal proteins (fraction III) were so treated to reduce denaturation, separated by SDS-PAGE and stained for heme to see if either of the enhanced proteins (50 kd and 54 kd) present in strain IIID were heme-containing. Upon staining with 3,3',5,5'tetramethyl benzidene three major heme positive regions appeared within the 48 - 68 kd region (Figure 5). These were designated largest to smallest respectively, P1 (64 kd), P2 (56 kd) and P3 (51 kd). On subsequent protein staining using Coomassie blue, these three bands plus a smaller fourth band, P4 (47 kd), showed up very strongly. The molecular weights indicated are larger than what would be seen on a denaturing gel due to the added porphyrin group which accounts for 650 daltons (Nebert et al, 1982) which was not lost in this method of sample preparation. P1, P2, P3 and P4 were electroeluted and concentrated from gel slices cut from a partially heme-stained gel. When P2 and P3 electroeluted proteins were examined on an SDS polyacrylamide denaturing gel alongside strain IIID CHAPS solubilized and column purified microsomal proteins (fraction III) they migrated to the same position as the two enhanced proteins of 50 kd and 54 kd found in strain IIID (Figure 4). This confirmed that these two enhanced proteins were heme-containing, further supporting their identity as cytochrome P450s.

Figure 5: Heme and protein stain of strain IIID microsomal proteins (fraction III). Protein (300 µg) was appplied to the gel and stained for heme. Heme positive areas were marked, the gel was destained and restained for protein with Coomassie blue. The three heme staining proteins P1, P2 and P3 and the fourth protein P4 visible upon protein staining are indicated. Reprinted from Houpt et al (1988).


PROTEIN
HEME
68
58
48
30
P1
P2
P3
P4

3.2.5 Cytochrome P450 content

Total cytochrome P450 content, measured using CO difference spectra, indicated a substantial increase in cytochrome P450 in strain IIID when compared to the nonresistant strain Canton S (Table 1 and Figure 6). This increase was as much as five fold and could be correlated with the increase in proteins P2 and P3 found in enhanced amounts in strain IIID.

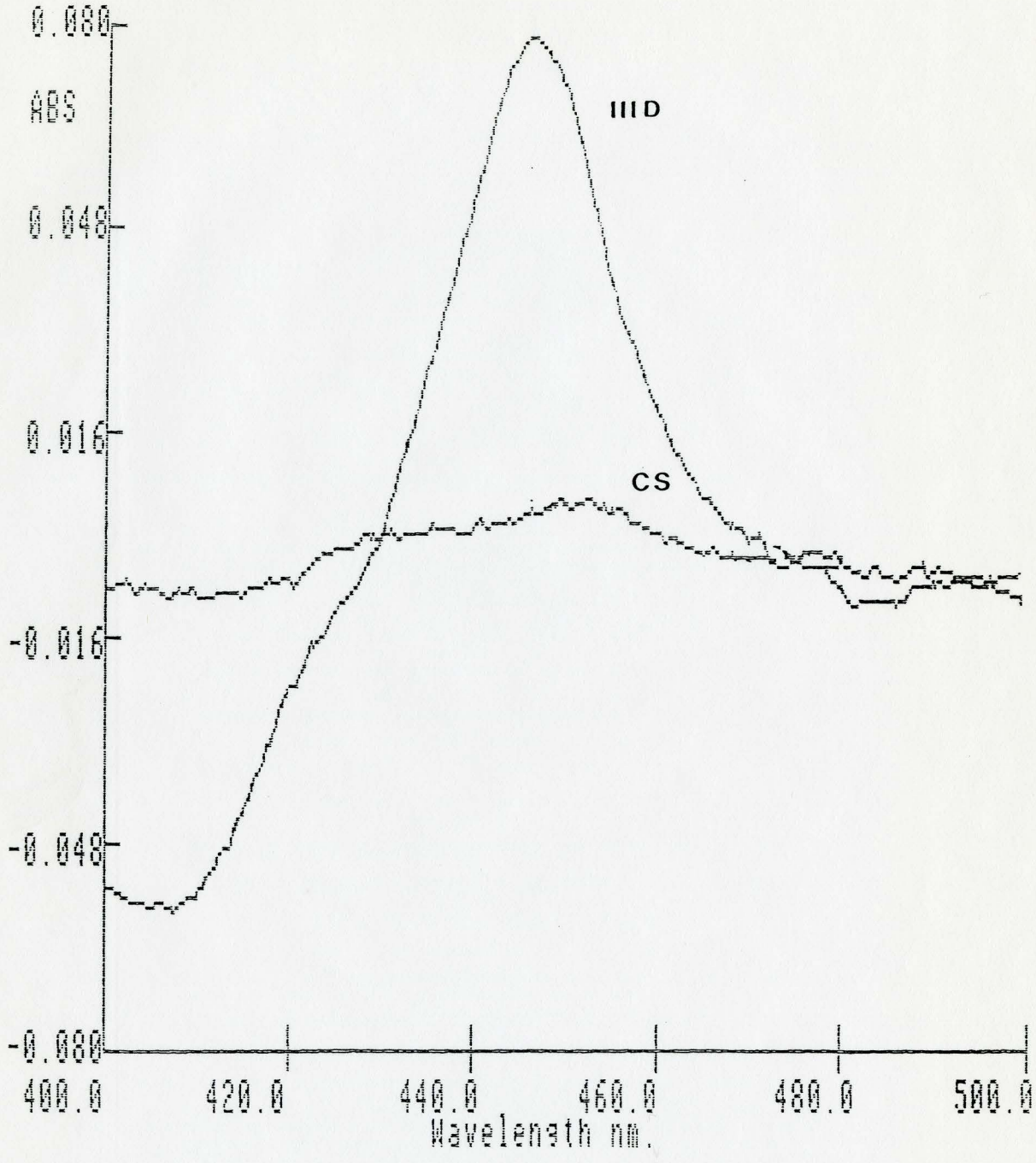
3.3 Analysis of Strains with Antibody

3.3.1 Antibody production

Cytochrome-P450-directed antibodies were produced in a male New Zealand white rabbit using CHAPS solubilized column purified (fraction III) microsomal proteins from strain IIID. One initial injection and two subsequent booster injections were administered. Serum was collected in large quantities at two time points; one week after the primary boost (Ab1) and when the rabbit was sacrificed one and one half weeks after the secondary boost (Ab2).

In order to determine the specificity of the Ab2 serum, its effect on cytochrome P450 content was examined by CO difference spectra. It was found however, that as increasing amounts of serum were added to the microsomal sample in an attempt to titer out cytochrome P450, the apparent amount of cytochrome P450 increased (data not shown). It was assumed that this apparent increase was in fact due to CO-related, differential

Figure 6: CO difference spectra of CHAPS solubilized microsomal proteins (70 µg) from Canton S (CS) or strain IIID. Cytochrome P450 contents were Canton S, 1.9 nmole/mg protein; strain IIID, 13.2 nmole/mg protein.


0.080
ABS
0.048
0.016
-0.016
-0.048
-0.080
IIID
CS
400.0
420.0
440.0
460.0
480.0
500.0
Wavelength nm.

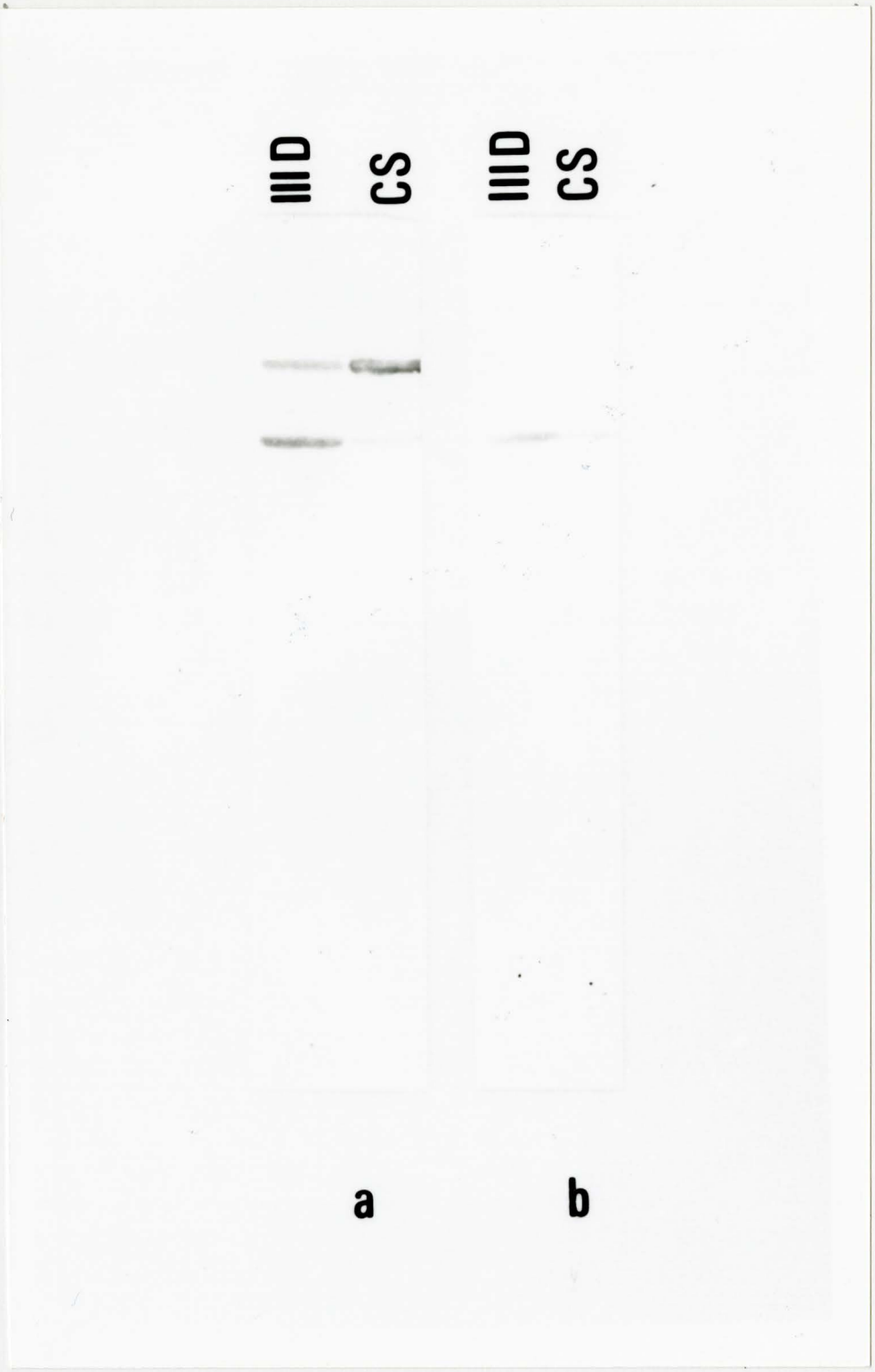
light absorption caused by hemoglobin present in the serum. For this reason Ab2 was subjected to ammonium sulphate precipitation and DEAE column chromatography to isolate the IgG fraction and remove any constituents that might interfere with characterization assays. This Ab2-IgG fraction was subsequently successfully characterized (section 3.3.3).

Serum collected after the primary boost (Ab1) was subjected to a Canton S Sepharose column in order to remove antibodies common to both Canton S and IIID. On a Western immunoblot of Canton S and strain IIID CHAPS solubilized microsomal proteins, untreated Ab1 was reactive to two proteins, one common to both strains and one found in great excess in strain IIID (Figure 7). A number of minor proteins could also be detected. After treatment with Canton S proteins, the serum (Ab1-B) remained reactive only to the protein found in excess in IIID. Hence common antibodies were successfully removed and the blocked serum was specific for strain IIID. Ab2-IgG was also treated in a similar manner with Canton S proteins attached to a Sepharose column however removal of common antibodies did not occur. This was probably due to insufficient regeneration of the column.

3.3.2 Immunoanalysis of Microsomal Proteins

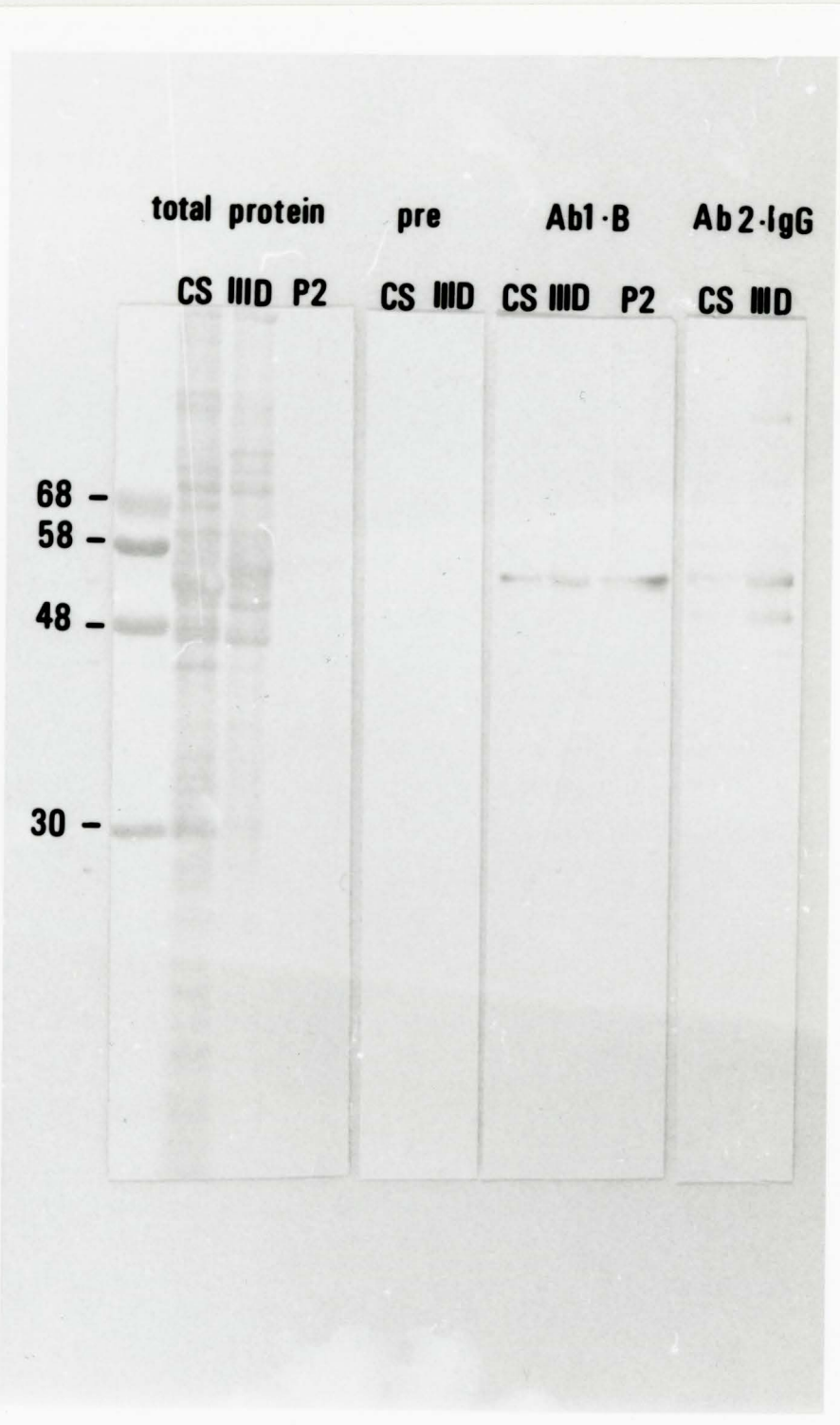
Western immunoblots of Canton S and strain IIID CHAPS solubilized microsomal proteins were immunostained with either

Figure 7: Western immunoblot of Canton S and strain IIID microsomal proteins stained with either (a) untreated serum Abl or (b) Canton S blocked serum Abl-B as the primary antibody. Antigenic proteins were visualized with alkaline phosphatase linked second antibody and NBT/BCIP substrate.


IIID
CS
IIID
CS
a
b

Abl-B or Ab2-IgG to determine the specificity of the antibodies (Figure 8). A total protein stain of the immunoblot clearly indicated the presence of P2 (54 kd) and P3 (50 kd) in strain IIID and the 52 kd protein in Canton S. Gel isolated P2 (section 3.2.4) migrated to the same position as P2 in strain IIID samples. Preimmune serum obtained from the rabbit prior to injection with antigen gave no reaction on the Western blot in either Canton S or IIID. The Canton S purified serum (Abl-B) reacted primarily with only one polypeptide, present in both strains but to a much larger degree in strain IIID. This polypeptide migrated to the same point as gel isolated P2, which also stained positively with Abl-B. Ab2-IgG was reactive mainly to two proteins that migrated to similar positions as P2 and P3 in the total protein stain. Again greater antigenic responses were observed in strain IIID when compared with Canton S. Other proteins were also reactive to a lesser extent towards Ab2-IgG including P1 and P4. These immunoblots suggest that antibodies were produced towards P2 and P3, both of which were previously determined to be putative cytochrome P450s. Two distinct serums were available, one immunogenic towards P2 (Abl-B) and one immunogenic towards P2 and P3 (Ab2-IgG). Ab2-IgG was used to screen the lambda gt11 expression library as it was immunogenic to both proteins of interest, P2 and P3. The slight difference in immunogenicity between the two serums was useful as clones obtained using Ab2-IgG

Figure 8: Western immunoblot of Canton S and strain IIID microsomal proteins stained for total protein with amido black or immunostained with preimmune serum, Ab1-B or Ab2-IgG as indicated. Detection occurred with alkaline phosphatase linked second antibody and NBT/BCIP substrate.


total protein
pre
Ab1·B
Ab2·IgG
CS IIID P2
CS IIID
CS IIID P2
CS IIID
68 –
58 –
48 –
30 –

in an initial screen could be characterized as sharing epitopes with P2 if they were also immunogenic to Abl-B.

3.3.3 Characterization of the Antibody

Ab2-IgG was determined to remove cytochrome P450 from strain IIID microsomal fractions as detected by CO difference spectra. Antibody (7 mg) was incubated with .3 nmole cytochrome P450 at room temperature for 0, 1 or 16 hours. Antibody / antigen complexes were removed by centrifugation and the supernatants were assayed for cytochrome P450 content. Over the 16 hour incubation period the amount of cytochrome P450 present decreased to 53% of the original (Figure 9). There was no significant decrease in a control to which no antibody had been added. The fact that cytochrome P450 was not removed entirely could have been due to insufficient quantity of antibody or the presence of other cytochrome P450 species not reactive towards the antibodies in Ab2-IgG.

The precipitated antibody/antigen complexes from the characterization assay were washed, resuspended and electrophoresed on denaturing gels. Silver staining indicated the presence of P2, P3 and P4 proteins in the precipitated samples treated with antibody (Figure 10). Upon denaturation, the IgG in these complexes separated into its component light and heavy chains. The molecular weight of the rabbit light chain is 23 kd, and of the heavy chain is 50 kd (Nisonoff et al, 1984). The heavy

Figure 9: Effect of Ab2-IgG on cytochrome P450 content in strain IIID CHAPS solubilized microsomal samples. After 1 or 16 hours incubation in the presence (Δ) or absence (O) of Ab2-IgG, samples were centrifuged and supernatants were assayed for cytochrome P450 content with CO difference spectra. % unprecipitated cytochrome P450 is plotted against time of incubation.

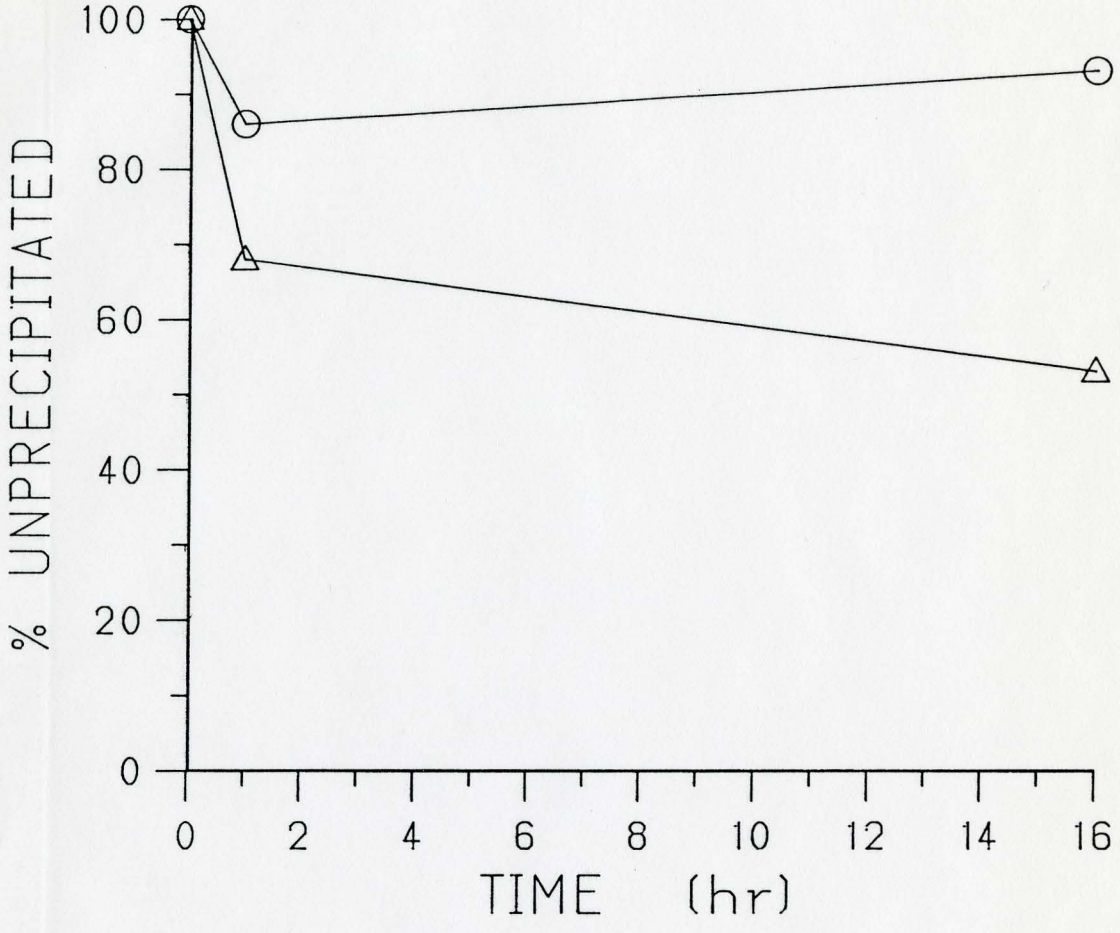
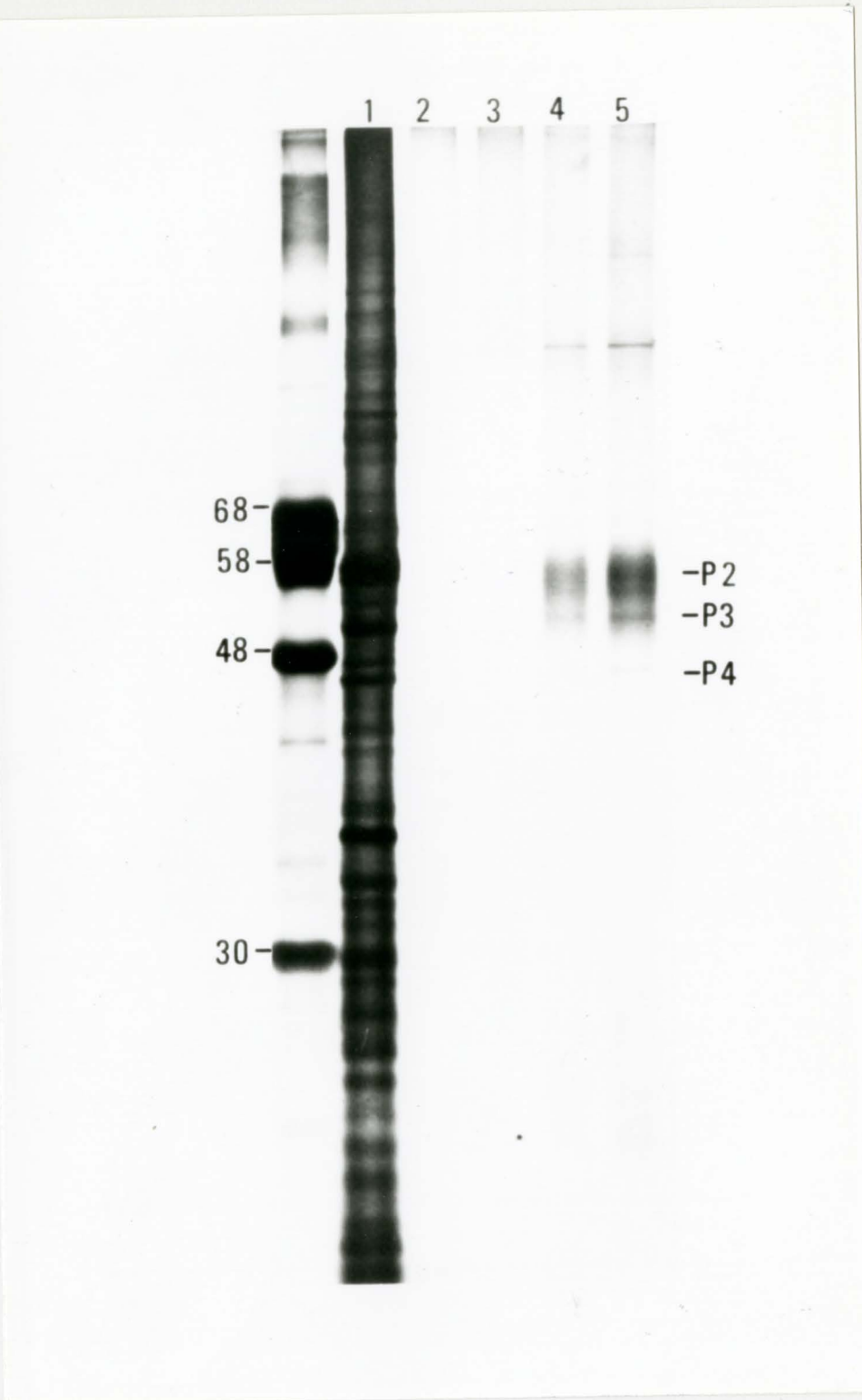

% UNPRECIPITATED
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
TIME (hr)

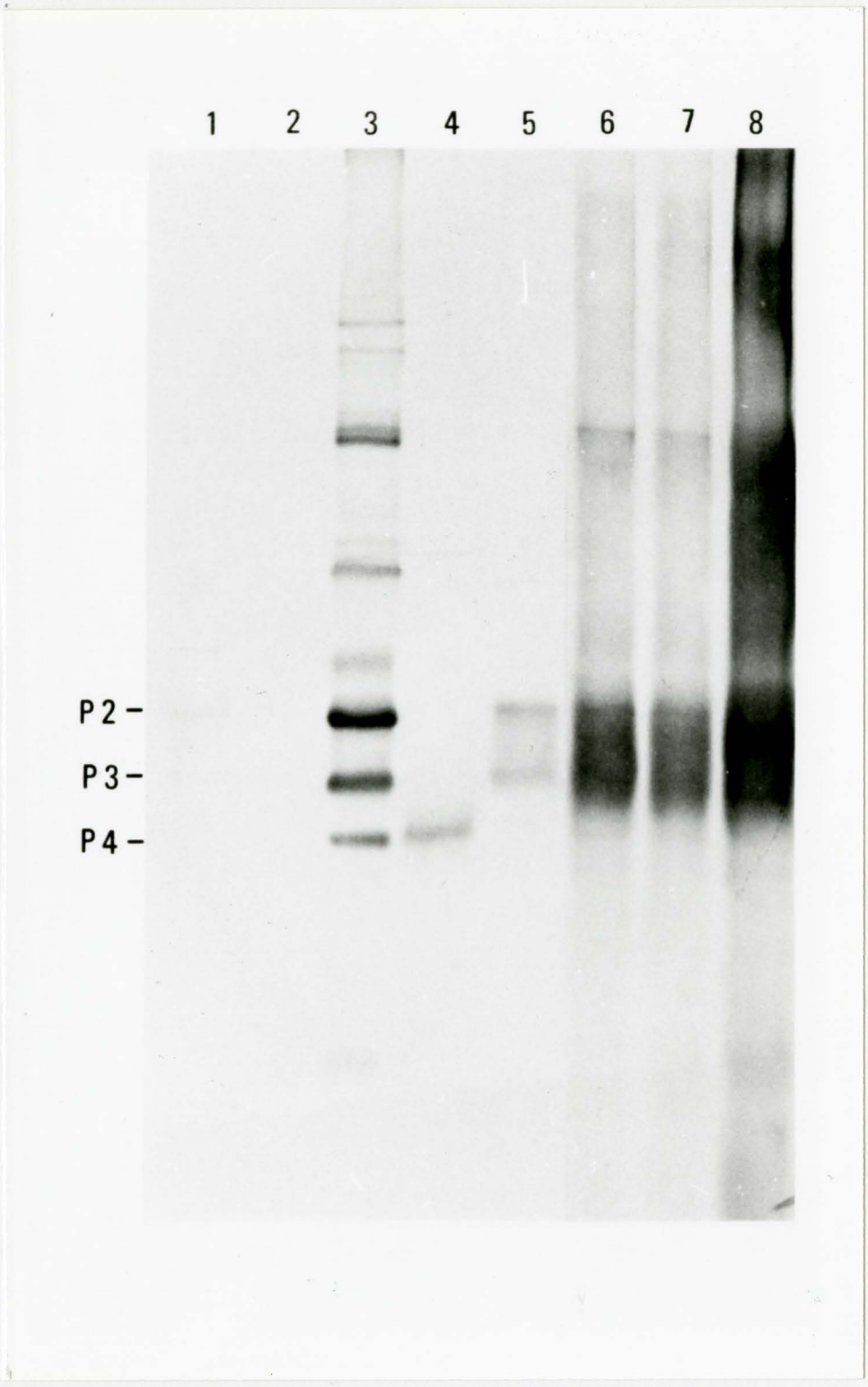
Figure 10: SDS polyacrylamide gel, stained for total protein, showing material pelleted after incubation with Ab2-IgG (lanes 4 and 5) or without Ab2-IgG (lanes 2 and 3) for 1 hour (lanes 2 and 4) or 16 hours (lanes 3 and 5). Lane 1 contains CHAPS solubilized IIID microsomal proteins. The positions of P2, P3 and P4 are indicated.


1
2
3
4
5
68
58
48
30
-P2
-P3
-P4

chain interfered with staining in the 45 - 55 kd region resulting in smearing, however distinct microsomal protein bands could be seen. Small amounts of protein were also visible in the control samples with no added antibody however they were insignificant in quantity when compared to the antibody containing samples. A similar gel was Western blotted and immunostained using Ab2-IgG as the primary antibody (Figure 11). Proteins P2, P3 and P4 were detected. The heavy chains of the precipitated antibody were also detected due to binding with the second antibody (alkaline phosphatase linked goat anti-rabbit IgG) again resulting in a smear in the 45 - 55 kd region. An attempt was made to block the IgG heavy chains on the filter by preincubating the filter with horse radish peroxidase goat anti-rabbit IgG at a low dilution. Subsequent staining with the alkaline phosphatase linked second antibody resulted in smearing in this region suggesting some IgG heavy chains were not blocked with the horse radish peroxidase antibody. When IgG alone was examined by immunostaining only one diffuse band appeared in the region, confirming the distinct bands seen previously were due to microsomal proteins precipitated by the IgG. Although the results were not as difinitive as one might have wished, they did support the idea that Ab2-IgG contained cytochrome P450 directed antigenic determinants.

In collaboration with Nicholas Okoampah, the correlation between mixed function oxidase activity and antigenic response was

Figure 11: Western immunoblot, preblocked with horse radish peroxidase conjugated goat-anti-rabbit IgG antibody, then stained with Ab2-IgG, showing CHAPS solubilized strain IIID microsomal material pelleted after incubation with Ab2-IgG (lanes 6 and 7) or without Ab2-IgG (lanes 1 and 2) for 1 hour (lanes 2 and 7) or 16 hours (lanes 1 and 6). Lane 3 contains strain IIID CHAPS solubilized microsomal proteins. Lanes 4 and 5 contain gel isolated P4, and P2 and P3, the positions of which are indicated. Lane 8 contains Ab2-IgG.


1
2
3
4
5
6
7
8
P2 –
P3 –
P4 –

examined. Crude fly extracts from several wildtype and resistant laboratory stocks and a number of homozygous chromosome III strains derived from natural populations (a gift of Dr. C.F. Aquadro, Section of Genetics and Development, Cornell University) were assayed for mixed function oxidase activity using the 7-ethoxycoumarin assay. The fly homogenates were also electrophoresed on 10% SDS polyacrylamide gels, elecroblotted onto nitrocellulose and immunostained with Abl-B. Densitometry was used to determine the antigenic response of P2 and three other control proteins. Figure 12 shows the relationship between mfo activity and the antigenic response of P2 and one of the control proteins. A positive correlation was observed between antigenic response to P2 and mfo activity. The control protein and the other two proteins which were not included in Figure 12 showed no correlation. The results of this experiment support the hypothesis that Abl-B is directed towards a protein -- probably cytochrome P450 -- involved in the mfo system.

3.4 Isolation of Lambda gtll Clones

3.4.1 Preparation of Lambda gtll with Drosophila cDNA inserts

The expression vector lambda gtll produces β-galactosidase fusion proteins which can then be screened for antigenicity towards an antibody. cDNA is inserted into an EcoRl site near the 3' end of the β-galactosidase gene close to the translation termination codon (Figure 13b). A Drosophila lambda gtll

Figure 12: The relationship between antigenic response of fly extract proteins and 7-ethoxycoumarin specific activity for nine different Drosophila melanogaster strains. A control protein (O) and protein P2 (Δ) were compared. Canton S and strain IIID responses are indicated.

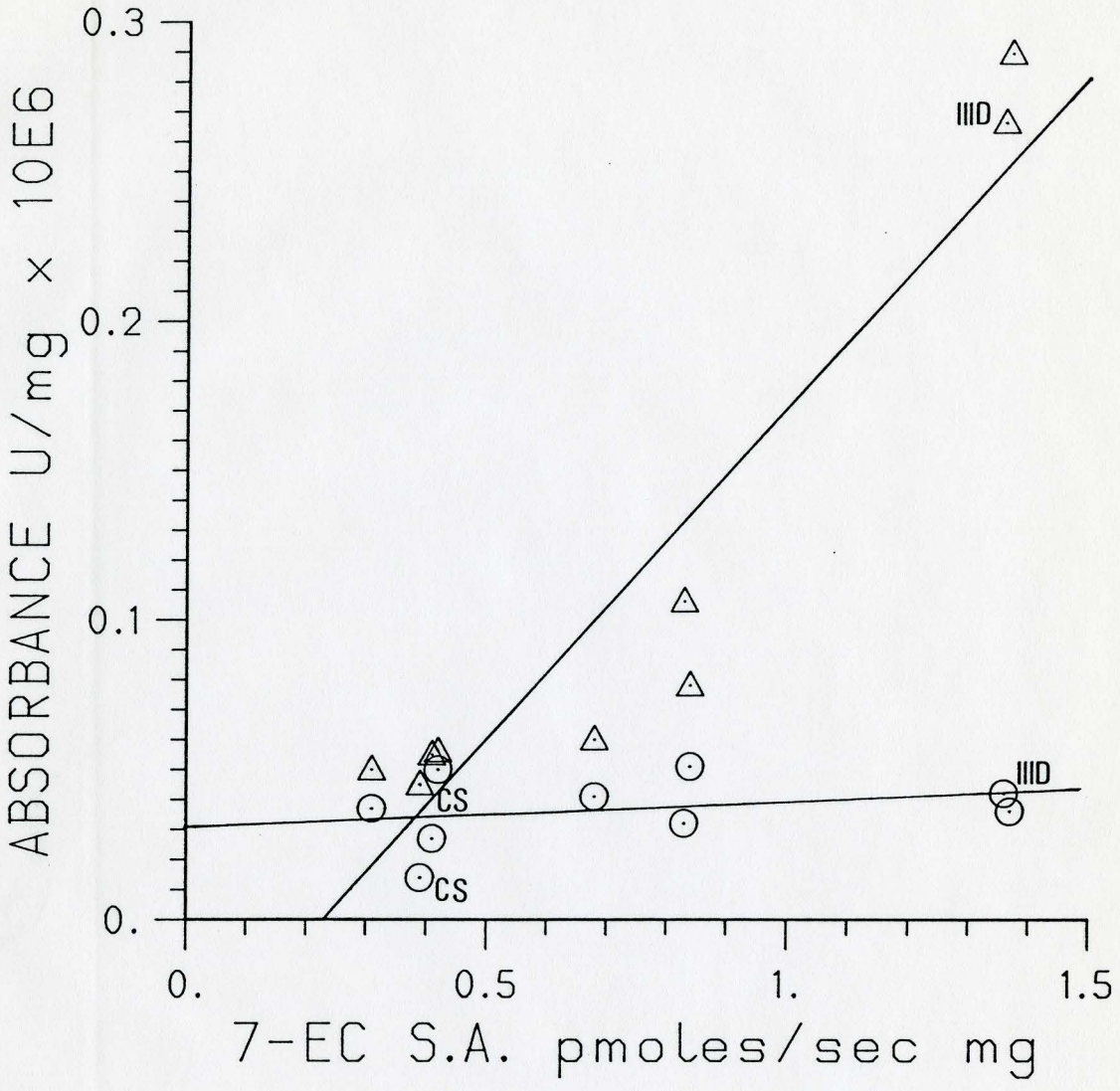
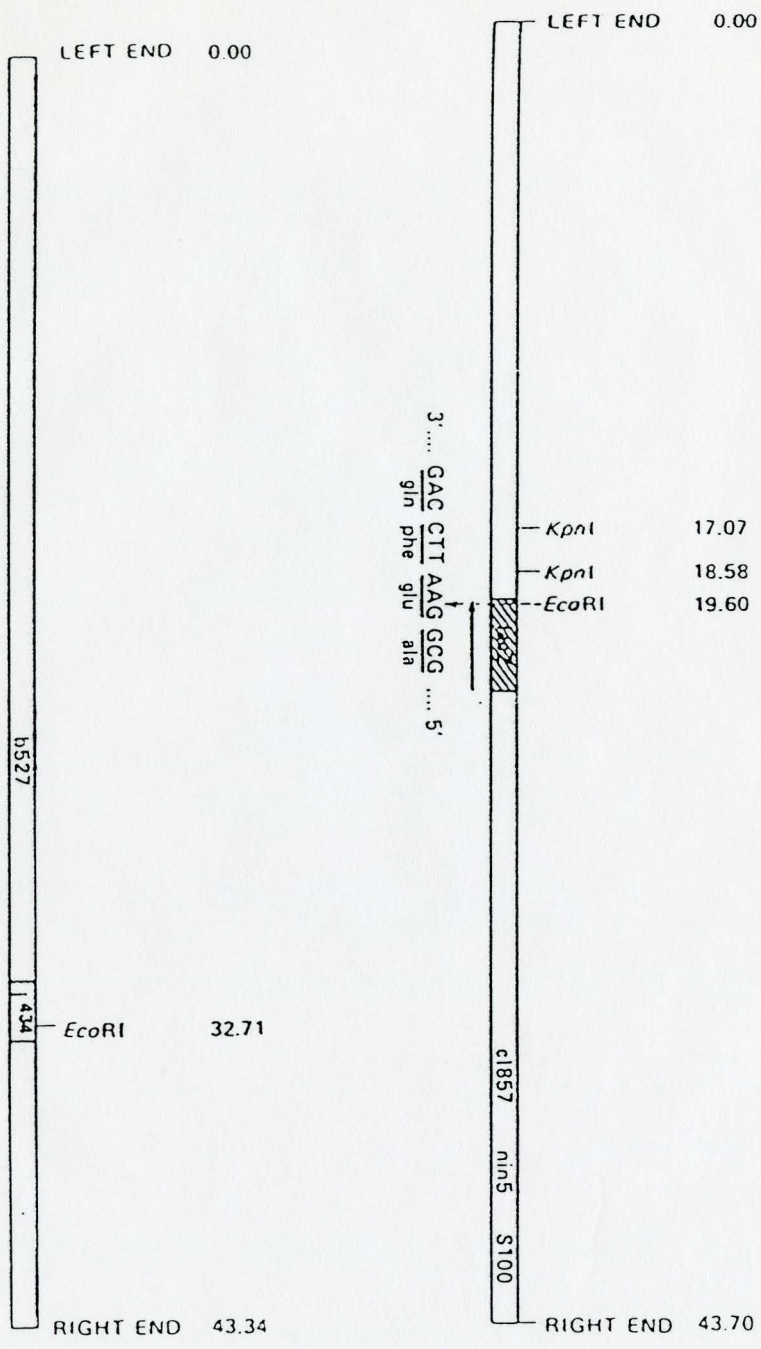

ABSORBANCE U/mg x 10E6
0.3
0.2
0.1
0.
0.
0.5
1.
1.5
7-EC S.A. pmoles/sec mg
IIID
IIID
CS
CS

Figure 13: (a) Lambda gt10 cloning vector showing the EcoRl restriction site. (b) Lambda gt11 cloning vector showing Kpnl and EcoRl restriciton sites. Distance from the left end in kilobases is indicated to the right of each vector. Modified from Huynh et al (1986).


LEFT END 0.00
b527
cI434
EcoRI 32.71
RIGHT END 43.34
a
LEFT END 0.00
3'.... GAC CTT AAG GCG .... 5'
gln phe glu ala
KpnI 17.07
KpnI 18.58
EcoRI 19.60
cI857
nin5
S100
RIGHT END 43.70
b

expression library was produced by ligating cDNA inserts obtained from a lambda gt10 (Figure 13a) library into lambda gt11 arms avoiding the need to make cDNA from mRNA, a long and tedious process. Lambda gt10 libraries from various stages of wildtype Drosophila melanogaster were obtained from Dr. T. Kornberg (Department of Biochemistry and Biophysics, University of California, San Fransisco). For the purpose of preparing the lambda gt11 library, lambda gt10 libraries containing cDNA inserts from male and female adult flies and larval stages were used. These particular stages were chosen because of their high cytochrome P450 levels. During the pupal stage it has been shown cytochrome P450 content is low (Hodgson, 1983), probably because it is a nonfeeding stage (Morton, personal communication). The chance of recovering a clone containing cDNA for a gene not expressed during that stage, therefore, would be slim.

In order to obtain enough lambda gt10 phage to produce sufficient quantities of insert cDNA the libraries were amplified on large Petrie plates before harvesting the phage and preparing DNA. There was a risk that the insert of interest (cytochrome P450) was contained in a poorly growing recombinant which would be selected against in the amplification process. Lambda gt10 is capable of carrying inserts up to 7.6 kb in size (Huynh et al, 1986). Upon EcoR1 digestion, inserts from lambda gt10 DNA, pooled from the various Kornberg libraries used, were released and were

easily separated from the two lambda arms (sizes 32.7 kb and 10.6 kb) by agarose gel electrophoresis followed by isolation from the gel with Geneclean. Approximately 200 ng of lambda gt10 insert cDNA was ligated into 1 µg lambda gt11 arms. DNA was packaged *in vitro* into phage particles and plated onto a modification$^+$ / restriction$^-$ host. By plating the phage in the presence of the gratuitous inducer IPTG and the β-galactosidase substrate X-gal it was possible to determine the percentage of clones containing inserts. If the β-galactosidase gene was interrupted with an insert, plaques were clear on indicator plates. No inserts resulted in blue plaques indicating an uninterrupted β-galactosidase gene product that was capable of cleaving the substrate, forming a blue precipitate. The *Drosophila* lambda gt11 library produced in this study contained a total of $1x10^5$ phage, of which 43% contained inserts, at a titer of $2x10^5$/ml.

3.4.2 Screening the lambda gt11 expression library

The *Drosophila* lambda gt11 library was screened using Ab2-IgG. This antiserum was directed mainly towards P2 and P3, as well as other minor proteins. Approximately 10^5 lambda gt11 phage ($4.3x10^4$ recombinants) were plated out on large LB plates and overlaid with nitrocellulose previously saturated with 10 mM IPTG to induce protein production from the β-galactosidase promotor. After 10-12 hours growth, sufficient protein accumulation had occurred allowing the filters to be probed. Clones containing

β-galactosidase fusion proteins that were reactive to Ab2-IgG were detected with alkaline phosphatase conjugated goat-anti-rabbit-IgG antibody and NBT/BCIP substrate. All plaques could be clearly seen on the stained filters. Those not reactive tended to have a donut shaped appearance while positive plaques were a solid purple colour (Figure 14). A total of 16 potentially positive plaques corresponding to reactive areas on the filter were picked from the original plates with the large end of a Pasteur pipette ensuring that the plaque of interest was obtained. Of the 16, ten were regrown, replated and rescreened in an attempt to confirm their positiveness. Three different clones were found to be reactive to Ab2-IgG (Figure 14). Clone gt11-A1 reacted very strongly to Ab2-IgG. Two other clones, gt11-A2 and gt11-C1 also reacted well with Ab2-IgG although only about one half as intensely as gt11-A1 (Figure 14). Each of these three clones were plaque purified, generally through three rounds of growth. Figure 15 describes the purification of gt11-A1. The positively stained plaque initially found on the large LB plate is seen in Figure 15a. Because the plug taken from this original plate contained 5-10 individual plaques, only 10% of the phage that were rescreened were positive (Figure 15b). An individual plaque picked from this first round of purification resulted in 99% positive plaques (Figure 15c). A negative plaque is indicated with the arrow. A third round of purification resulted in 100%

Figure 14: Antigenic response of lambda gt11 clones gt11-neg, gt11-A1, gt11-A2 and gt11-C1 using Ab2-IgG as the primary antibody followed by detection with alkaline phosphatase linked second antibody and NBT/BCIP substrate.

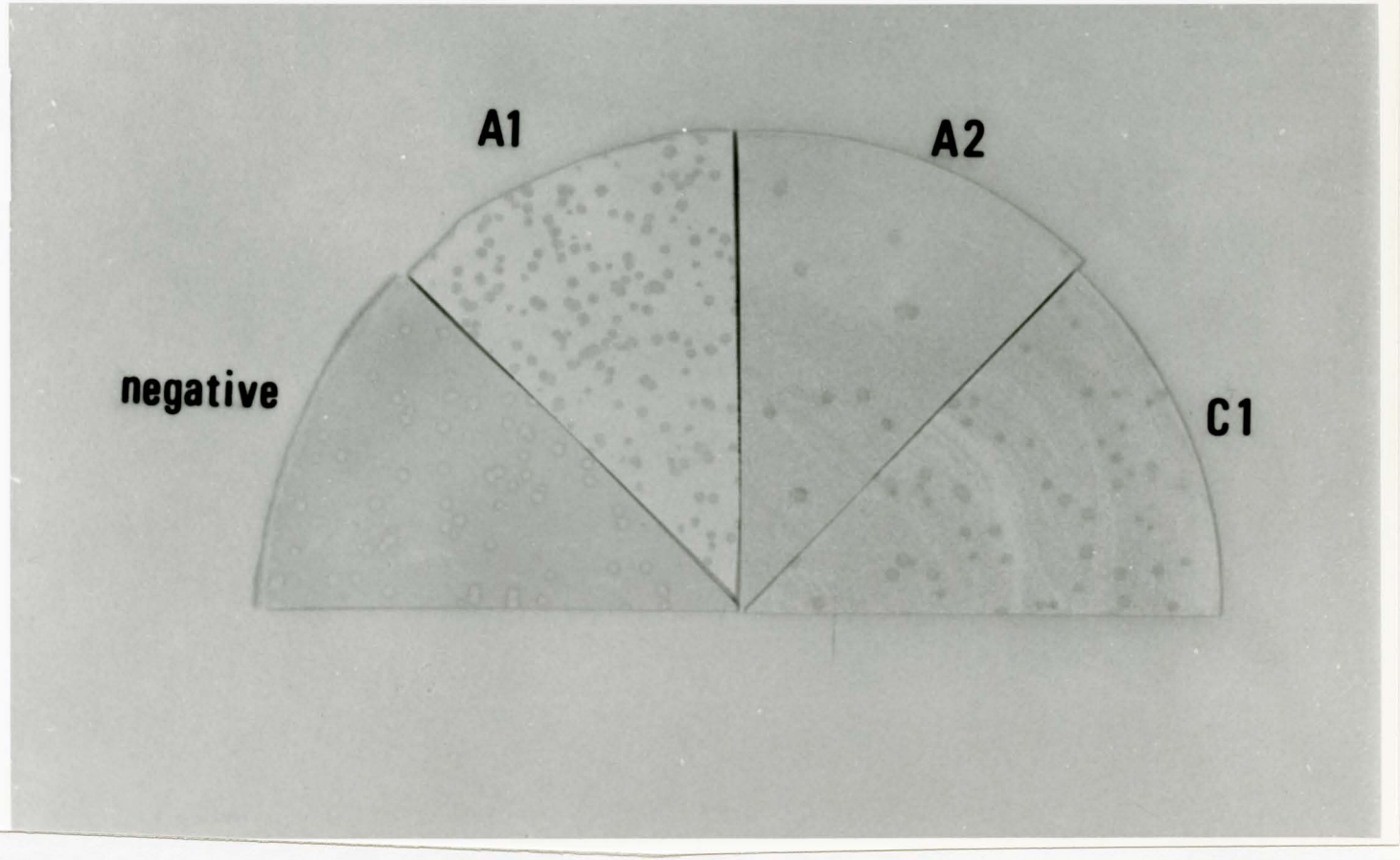
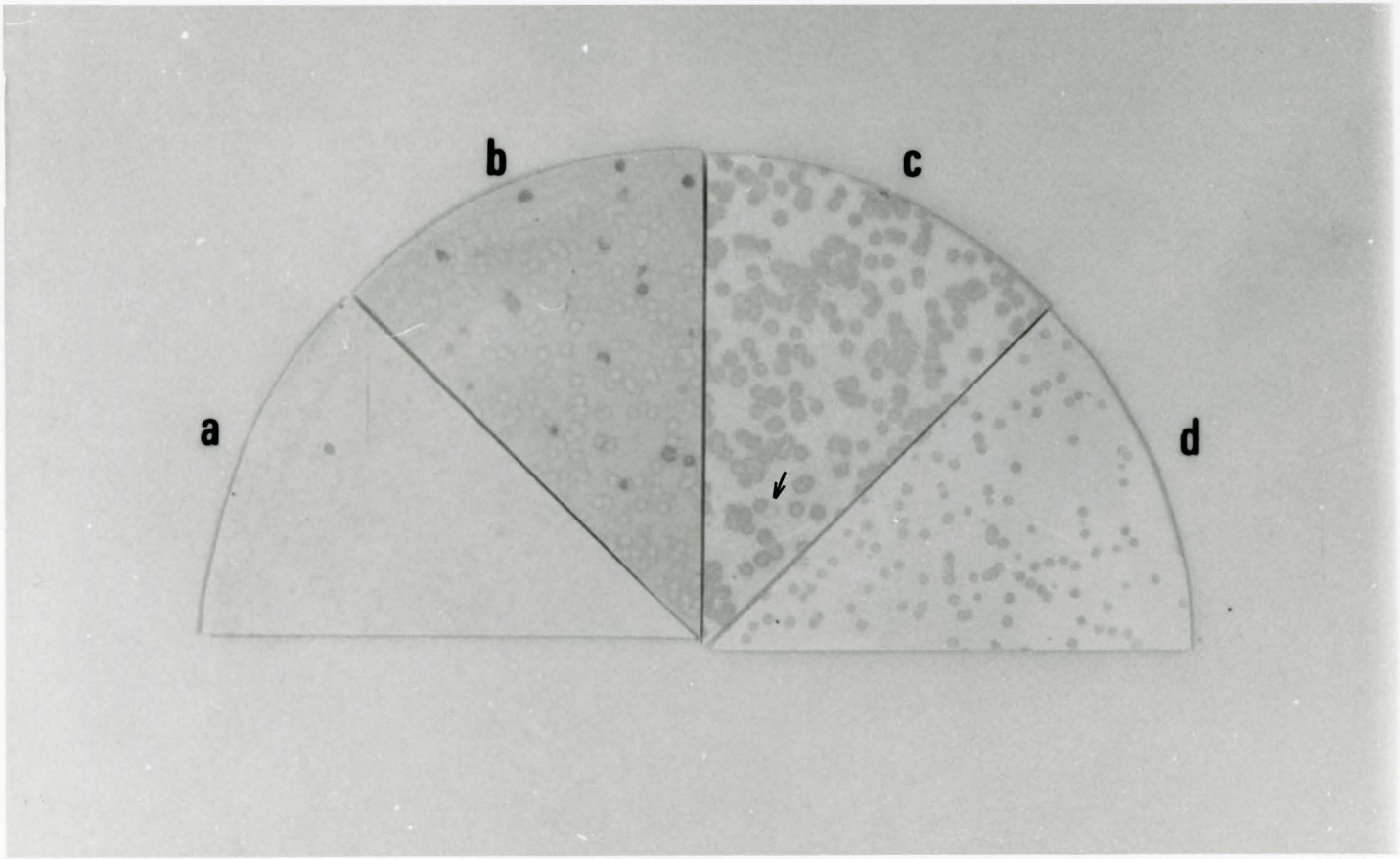

A1
A2
negative
C1

Figure 15: The purification of lambda clone gt11-A1. (a) the initial positive plaque observed; (b) after picking and rescreening the plug containing plaque "a"; (c) 99% positive phage from a plaque picked from "b". A negative plaque is indicated with the arrow; (d) pure lambda gt11-A1.


a
b
c
d

positive plaques (Figure 15d). The purified gtll-Al clone was stored at $4^{o}C$ and periodically tested to ensure it remained reactive. The clone was stable and always gave 100% positive plaques. Further work in this thesis concentrated on clone gtll-Al as this appeared to be the best candidate for a potential clone for cytochrome P450. As well, a negative clone (gtll-neg) containing no insert was picked as a blue plaque from an indicator plate for use as a negative control.

3.5 Analysis of Lambda gtll Clones

3.5.1 Preparation of Lysogens and Lysate Production

Lysogens were prepared from clones gtll-Al and gtll-neg by superinfecting E. coli Y1089 bacteria with phage. The rate of lysogen formation was very low, 3.3% for gtll-Al and 5.9% for gtll-neg, compared with published reports which suggested rates of 10 - 70% (Hyunh et al, 1985). The reason for this low yield is not known. Protein lysates were made from the lysogens produced, by inducing the temperature sensitive phage at $42^{o}C$, then inducing protein production from the β-galactosidase promotor with the addition of 10 mM IPTG. This resulted in the overproduction of either β-galactosidase in the case of gtll-neg or a fusion protein in the case of gtll-Al. Few E. coli proteins are larger than β-galactosidase hence fusion proteins are easily detected by SDS-PAGE (Huynh et al, 1986). Upon silver staining 5% SDS polyacrylamide gels containing lysogen lysates either induced or

uninduced (Figure 16), proteins were detected. β-galactosidase (116 kd) was detected in the gtll-neg lysogen in both the induced and surprisingly in the uninduced samples. This was most likely due to a leakiness of the plasmid pMC9 in E. coli Y1089 which contains $lacI^{Q}$, a repressor of the lac operon. Lysogen gtll-Al was successfully induced with the addition of IPTG and produced a fusion protein larger than β-galactosidase (Figure 16). Its molecular weight was calculated to be approximately 130 kd, of which 114 kd was the β-galactosidase portion, leaving the gtll-Al cDNA insert accounting for approximately 16 kd. This would correspond to a gtll-Al cDNA coding region size of 350 bp, assuming an average amino acid weight of 140 d. Large amounts of β-galactosidase were detected in gtll-Al, most likely due to degradation of the fusion protein. The gtll-Al fusion protein was very immunogenic to Ab2-IgG, the antibody used to screen the lambda gtll library (Figure 17a). Some E. coli proteins were also detected due to reactivity with anti-E. coli antibodies found in the rabbit serum. When an identical blot was immunostained with anti-β-galactosidase antibody (Figure 17b) a protein in the induced lysogen gtll-Al showed up at the same position as that in Figure 17a, indicating it was a fusion protein consisting of both a Drosophila polypeptide and a portion of β-galactosidase. Degradation of both β-galactosidase and the gtll-Al fusion protein as indicated by the presence of bands less than 116 kd in size was

Figure 16: Silver stained .5% SDS gel showing uninduced (lanes 1 and 3) and induced (lanes 2 and 4) lysates from E. coli Y1089 lysogens of gt11-neg (lanes 1 and 2) or gt11-A1 (lanes 3 and 4). The arrow indicates the gt11-A1 fusion protein observed in the induced sample.

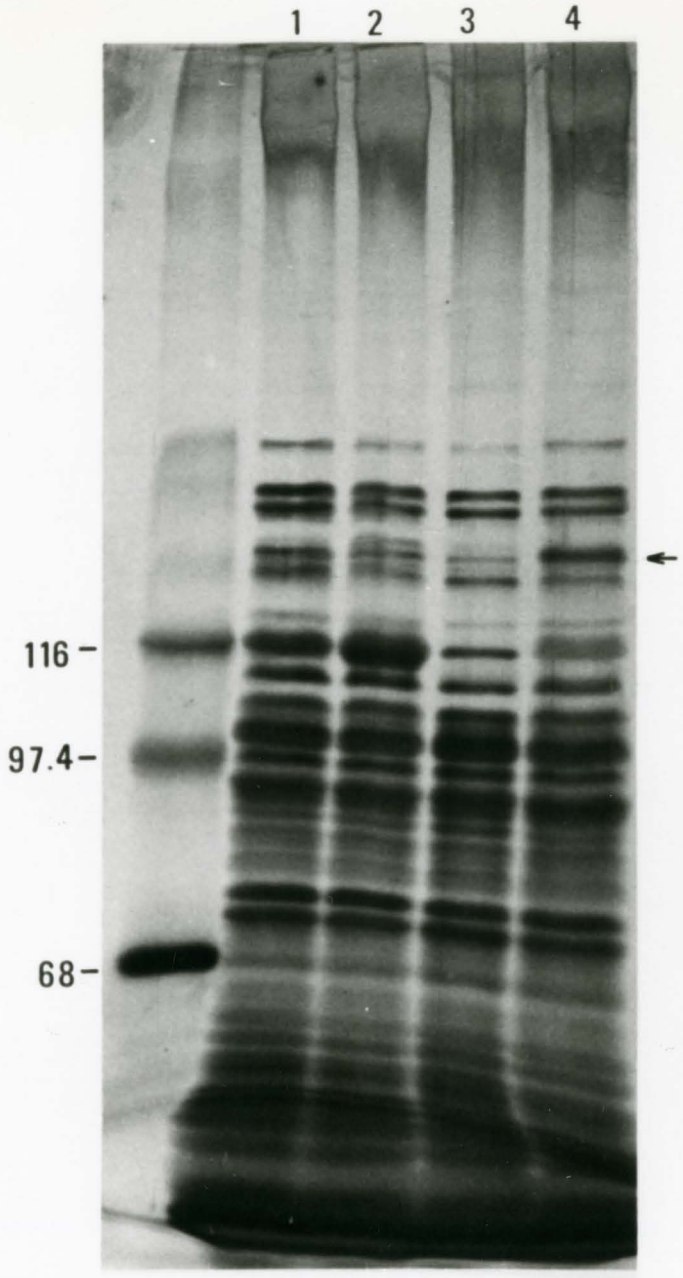
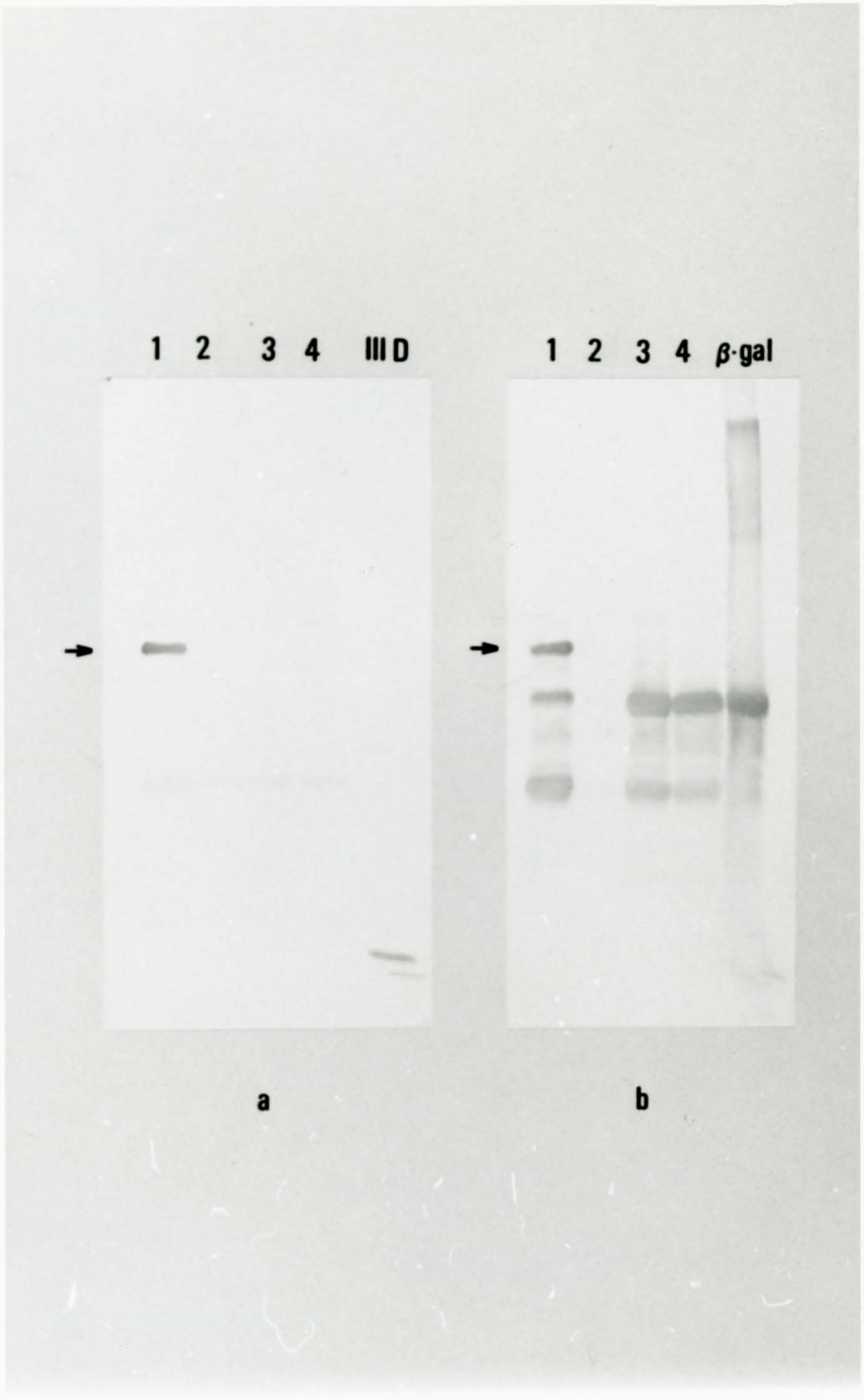

1
2
3
4
116
97.4
68

Figure 17: (a) Western immunoblot of uninduced (lanes 2 and 4) or induced (lanes 1 and 3) lysates from E. coli Y1089 lysogens of gt11-neg (lanes 3 and 4) or gt11-A1 (lanes 1 and 2) stained with Ab2-IgG as the primary antibody. (b) an identical blot stained with anti β-galactosidase as the primary antibody. Strain IIID CHAPS solubilized microsomes or β-galactosidase was applied in blots (a) or (b) respectively to serve as positive controls for the primary antibody. The arrows indicate the gt11-A1 fusion protein.


1 2 3 4 III D
1 2 3 4 β-gal
a
b

observed after staining the blot with the anti-β-galactosidase antibody.

3.5.2 DNA Preparation and Analysis

Phage DNA was isolated from all three immunogenic lambda gt11 clones and gt11-neg. Upon EcoR1 digestion and agarose gel electrophoresis it was determined that gt11-C1 contained a 1.8 kb insert and gt11-A2 contained a 2.0 kb insert (Figure 18). No insert was observed for clone gt11-A1 although all other experiments indicated that the β-galactosidase gene was interrupted with DNA. Because a small cDNA insert might not be detected under normal conditions, EcoR1 digested and endlabelled DNA from gt11-A1 was examined by polyacrylamide gel electrophoresis followed by autoradiography. Again no insert was observed (data not shown). The possibility that one of the gt11-A1 EcoR1 sites was destroyed during the cloning process was examined. It was known that one EcoR1 site existed because upon restricting gt11-A1 DNA and subsequently melting the lambda cos site, two DNA fragments of approximately 24.1 and 19.6 kb were detected with agarose gel electrophoresis. A more comprehensive study of the arm length was made by comparing the migration patterns of gt11-neg and gt11-A1 EcoR1 digested DNA on a .35% agarose gel. The two DNA fragments in EcoR1 digested gt11-A1 were closer together than the two fragments in gt11-neg (Figure 19). Careful visual observation indicated that the 19.6 kb fragment in

Figure 18: Ethidium bromide stained .5% agarose gel showing the EcoRl restriction patterns of lambda clones gtll-neg, gtll-Al, gtll-A2 and gtll-Cl. Molecular weight markers in kilobases are indicated.

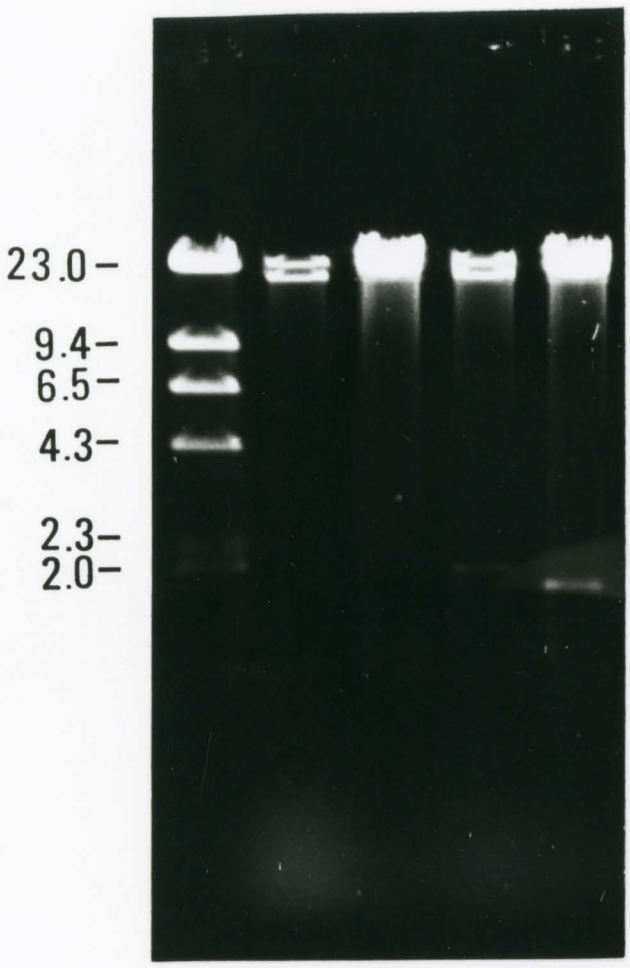
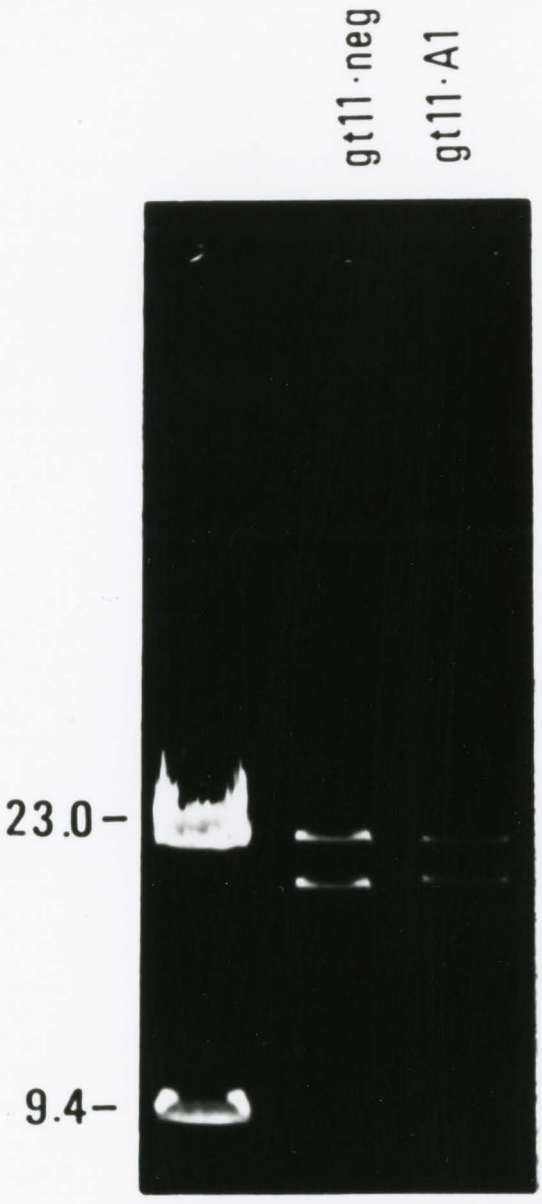

gt11·neg
gt11·A1
gt11·A2
gt11·C1
23.0
9.4
6.5
4.3
2.3
2.0

Figure 19: Ethidium bromide stained .35% agarose gel showing the EcoRl restriction patterns of lambda clones gtll-neg and gtll-Al.


gt11·neg
gt11·A1
23.0–
9.4–

gtll-Al was running slower than that of gtll-neg, indicating it was in fact larger than it should have been. This suggested that in gtll-Al the EcoRl site closest to the left end of the vector had been destroyed during the cloning process and that the more slowly migrating band was larger than normal because it also contained the cDNA insert. The restriction site nearest to the downstream side of this region was a Kpnl site located at 18.6, 1 kb from the destroyed EcoRl site. By digesting gtll-Al DNA with both Kpnl and EcoRl the insert could be removed along with 1 kb of lambda gtll DNA. The 1 kb Kpnl/EcoRl fragment from gtll-neg DNA would also be isolated to serve as a control for any experiments using the cDNA from gtll-Al as a DNA probe.

Kpnl/EcoRl double digests were made on gtll-neg and gtll-Al DNA and analysed by agarose gel electrophoresis (Figure 20). Clone gtll-neg DNA was resolved into fragments of 24.1, 17.1, 1.5 and 1 kb, exactly as expected (Figure 13b). Clone gtll-Al DNA contained fragments of sizes 24.1, 17.1 and 1.5 kb. The 1 kb fragment was absent as expected and in its place should have been some fragment larger than 1 kb, consisting of the 1 kb Kpnl/EcoRl fragment and the cDNA insert. When equal amounts of Kpnl/EcoRl digested DNA from gtll-neg and gtll-Al were loaded on a gel, the 1.5 kb fragment in gtll-Al DNA appeared approximately twice as intense as that in the gtll-neg DNA. This suggested that two DNA fragments comigrated at the 1.5 kb position in gtll-Al

Figure 20: Ethidium bromide stained .7% agarose gel showing the KpnI/EcoRI restriction patterns of lambda clones gt11-neg and gt11-A1. The presence and absence of the 1.0 kb fragment (indicated by the arrow) in clones gt11-neg and gt11-A1 respectively is seen.

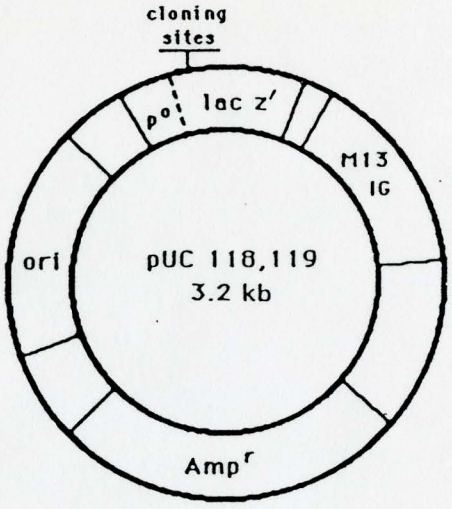
gt11·neg
gt11·A1
23.0
9.4
6.5
4.3
2.3
2.0

DNA. One of these fragments was the true KpnI/KpnI 1.5 kb fragment and the other was a .5 kb cDNA insert fused to the 1 kb KpnI/EcoRI fragment. This .5 kb insert corraborates earlier results with the fusion protein that indicated an insert of at least .35 kb should be present (section 3.5.1). The 1.5 kb KpnI/EcoRI A1 fragment and the 1 kb KpnI/EcoRI neg fragment were subcloned into pUC119. pUC119 was used instead of pUC118 primarily because of the orientation of the multicloning site (Figure 21). In pUC119 the EcoRI site in the multicloning site is closer to the sequencing primer than in pUC118. By inserting the gt11-A1 KpnI/EcoRI fragment into pUC119 the actual gt11-A1 cDNA insert could be easily sequenced without first having to sequence 1 kb of lambda gt11 DNA. Following ligation and transformation, mini plasmid preparations were made on insert-containing recombinants. Different clones were isolated containing either a 1 kb KpnI/EcoRI fragment from gt11-neg (pUC119-neg) or a 1.5 kb KpnI/EcoRI fragment from gt11-A1 (pUC119-A1) (Figure 22). The pUC119-A1 insert was used in the analysis of Drosophila genomic DNA and total RNA in Southerns and Northerns while the pUC119-neg insert was used as a control for the lambda gt11 DNA found in the pUC119-A1 insert.

3.5.3 Southern Analysis of Lambda gt11 Clones

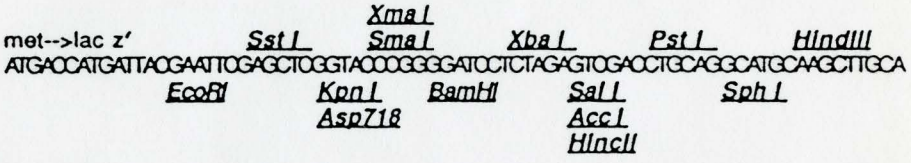
The pUC119-A1 KpnI/EcoRI insert was oligolabelled and used to probe a Southern blot containing DNA from various putative

Figure 21: Cloning vectors pUC118 and pUC119 showing the different orientations of the multicloning silte. Taken from Vieira and Messing (1987).



Cloning Sites

pUC 118



pUC 119

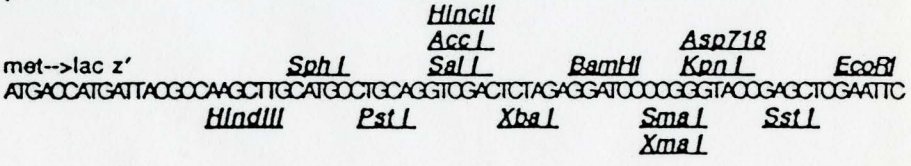
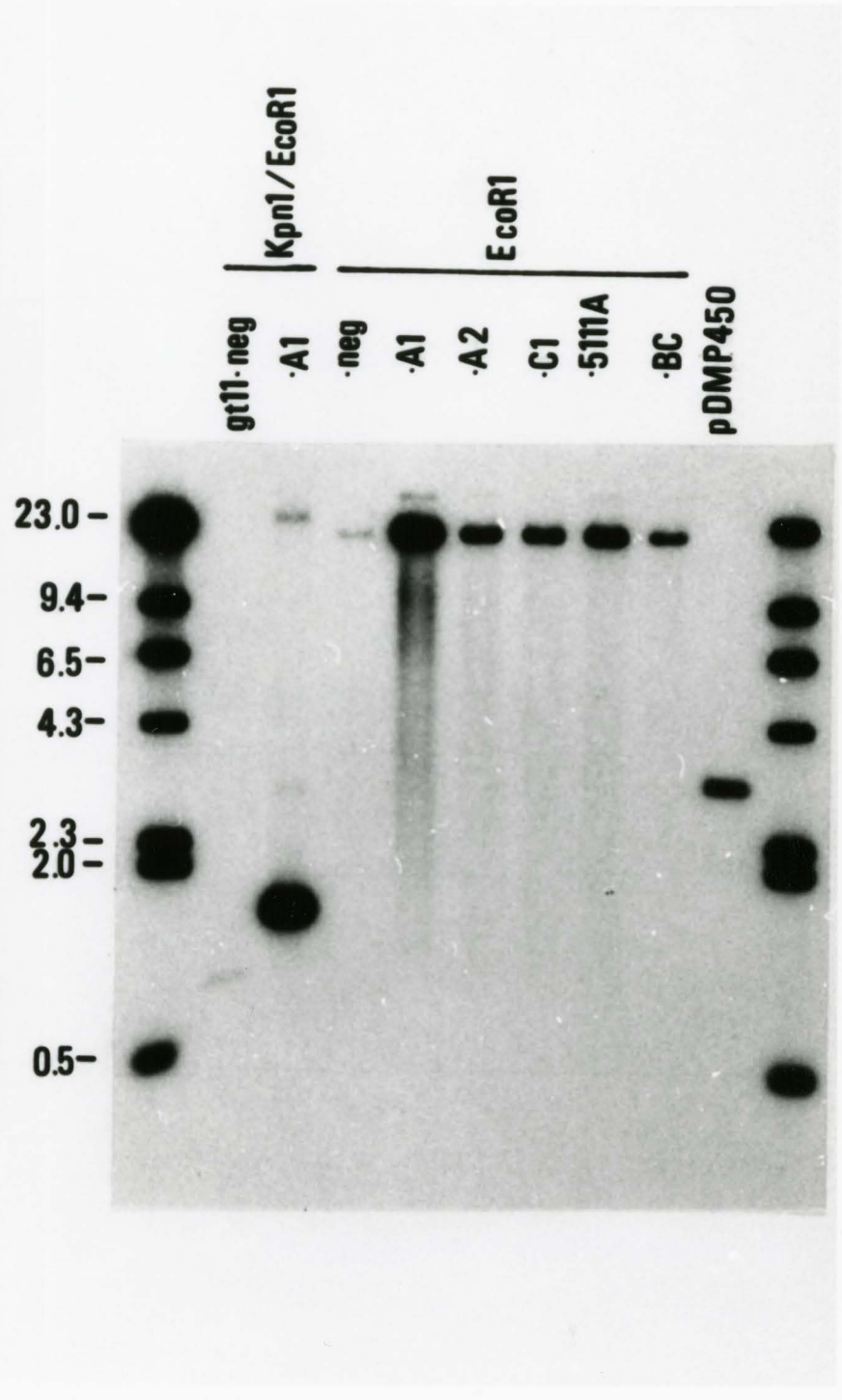


Figure 22: Ethidium bromide stained .7% agarose gel showing EcoRl restriction patterns of pUC119, pUC119-neg and pUC119-Al, and KpnI/EcoRl restriction patterns of pUC119-neg and pUC119-Al. The pUC119 vector DNA and the 1.0 and 1.5 kb inserts in pUC119-neg and pUC119-Al respectively are seen in the double digests.

pUC119
pUC119·neg
pUC119·A1
pUC119·neg
pUC119·A1
EcoR1
Kpn1/EcoR1
23.0
9.4
6.5
4.3
2.3
2.0

cytochrome P450 clones, isolated by this laboratory or obtained from other laboratories, to look for sequence similarity (Figure 23). At high stringency (68^{o}C) the pUC119-A1 insert bound to a 1.5 kb fragment from KpnI/EcoRI digested gt11-A1 DNA and the 1 kb KpnI/EcoRI fragment from gt11-neg DNA confirming the correct fragment had been subcloned into pUC119 and that it did contain lambda gt11 DNA. The probe hybridized to the 19.6 kb lambda gt11 arm in all lambda gt11 clones digested with EcoRI indicating the lambda DNA found in the pUC119-A1 probe originated from this arm. In a similar manner, hybridization occurred with the 23 kb fragment of the marker DNA, HindIII digested lambda DNA, from which lambda gt11 is derived (not obvious in figure 23). Inserts from gt11-A2 and gt11-C1, and gt11-BC (a gift of Dr. B. Cochrane, Department of Biology, University of South Florida) and gt11-5111A, (the latter two isolated independently of this study), did not hybridize to the pUC119-A1 insert DNA. pDMP450, a putative cytochrome P450 Drosophila clone with weak homology to a mouse cytochrome P450 cDNA was a gift of Dr. B. Cochrane. It contained a 2.1 kb insert that did not hybridize with pUC119-A1, although contaminating vector DNA (a 3.1 kb pUC derivative) did, possibly due to contamination of the pUC119-A1 insert probe with pUC119 vector DNA. When an identical Southern was probed at low stringency (42^{o}C), no additional bands appeared (data not shown), confirming

Figure 23: Autoradiogram, probed with the 1.5 kb KpnI/EcoRI insert from pUC119-A1, of Southern blot containing all laboratory clones containing putative cytochrome P450 genes. Lanes 1 and 2, KpnI/EcoRI digestion of gt11-neg and gt11-A1. Lanes 3 through 8 EcoRI digestions of the lambda gt11 clones indicated. Lane 9 EcoRI/HindIII digestion of pDMP450, described in the text. Note that very little lambda gt11-neg DNA was loaded on the gel. Endlabelled, HindIII digested lambda DNA molecular weight markers are indicated.


gt11-neg
-A1
-neg
-A1
-A2
-C1
-5111A
-BC
pDMP450
Kpn1/EcoR1
EcoR1
23.0
9.4
6.5
4.3
2.3
2.0
0.5

that of the five clones examined, none appeared to have significant sequence homology to gtll-Al.

3.6 Analysis of Drosophila genomic DNA and total RNA

3.6.1 Analysis of genomic DNA

The pUCll9-Al insert was used to probe genomic Drosophila DNA from both Canton S and IIID strains. Because pUCll9-Al contained lambda gtll DNA in addition to Drosophila cDNA the pUCll9-neg insert was initially used as a control. Dot blots of denatured genomic DNA were probed with both probes (Figure 24) at high stringency conditions (68^{o}C). Clone gtll-Al DNA was present as a positive control for each probe. In each case gtll-Al control DNA was detected, however genomic DNA hybridized only with the pUCll9-Al probe indicating that it was the cDNA portion of this probe not the lambda gtll portion that was binding to genomic DNA under these conditions. For this reason genomic Southerns were probed only with the pUCll9-Al insert probe.

Genomic DNA from Canton S and strain IIID, digested both singly and doubly with three six cutter enzymes (EcoRl, HindIII or BamHl) was Southern blotted and hybridized with the Kpnl/EcoRl insert from pUCll9-Al (Figure 25). The patterns observed for the two strains were identical for single digestions of the enzymes HindIII and BamHl. On EcoRl digestion however, a polymorphism was observed between Canton S and strain IIID. Strain IIID contained a faster migrating fragment than did Canton S. In some instances

Figure 24: Autoradiogram of genomic DNA dot blots of Canton S (CS) and strain IIID, probed with either pUC119-neg or pUC119-A1 insert. Lambda clone gt11-A1 DNA treated similarily served as a positive control for each probe.

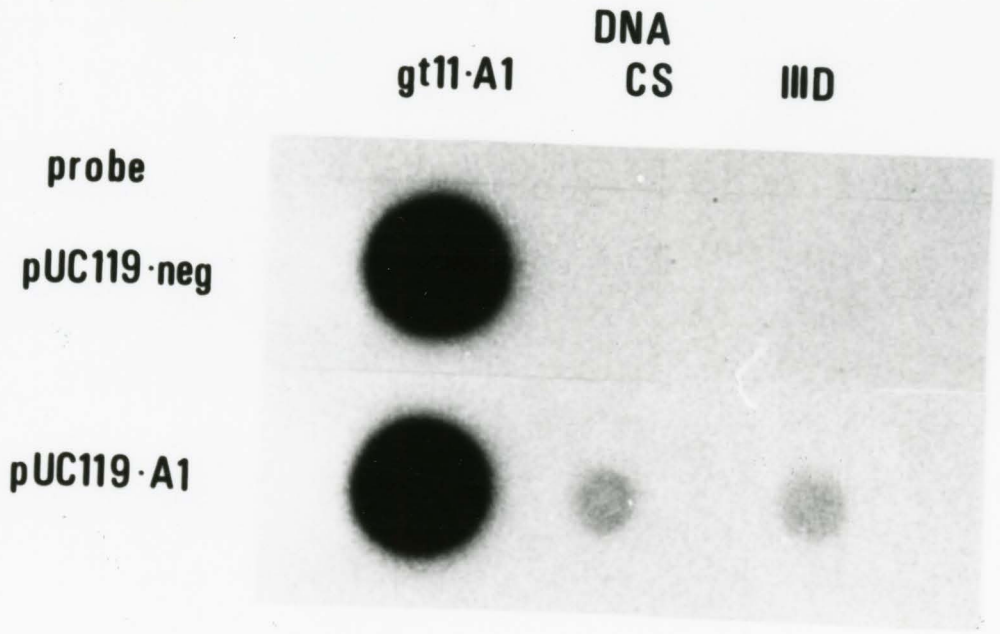
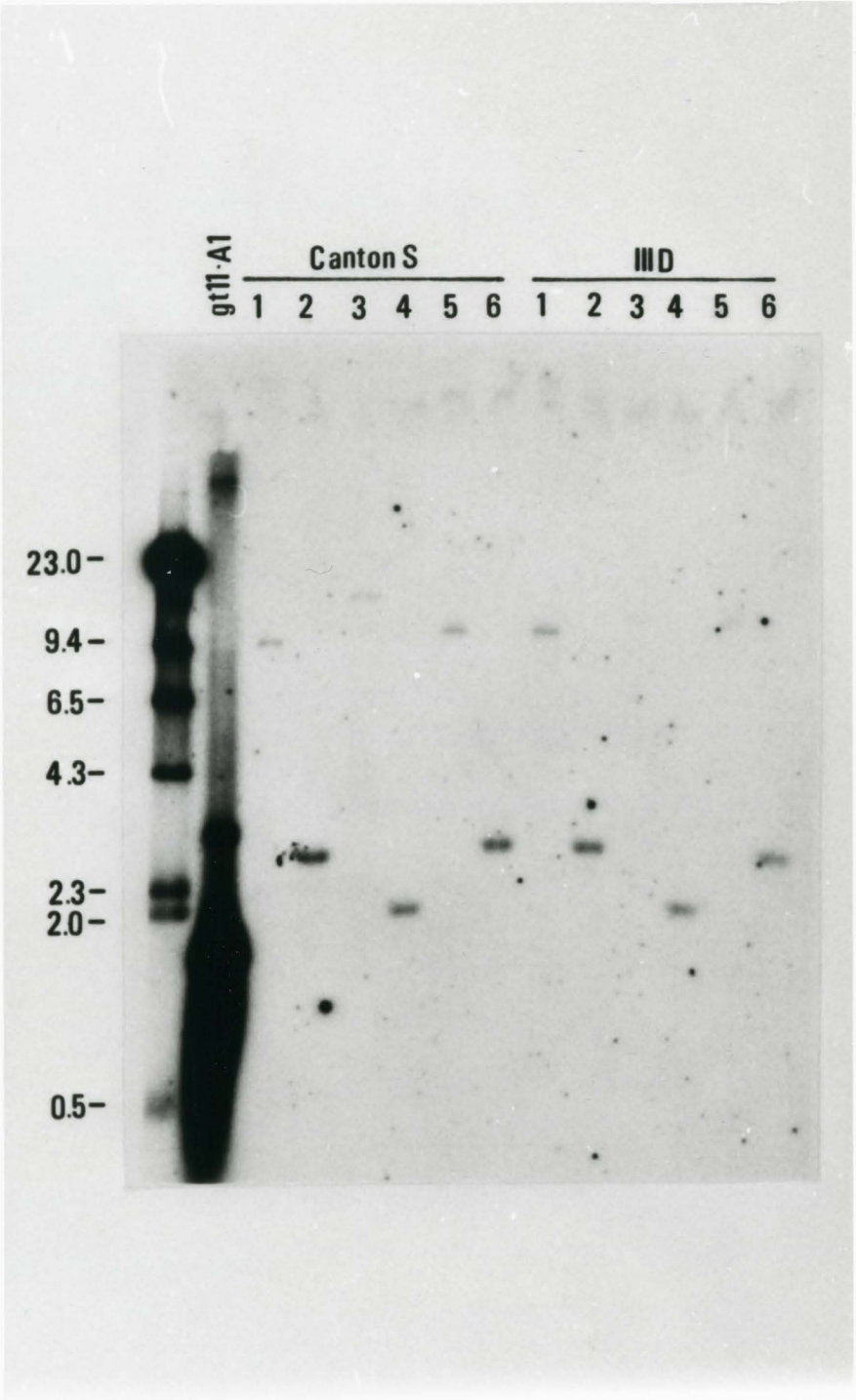

DNA
gt11·A1
CS
IIID
probe
pUC119·neg
pUC119·A1

Figure 25: Autoradiogram of Southern blot of Canton S and strain IIID genomic DNA digested with lane 1 BamHl, lane 2 HindIII, lane 3 EcoRl, lane 4 BamHl/HindIII, lane 5 BamHl/EcoRl, lane 6 HindIII/EcoRl and probed with the 1.5 kb Kpnl/EcoRl fragment from pUC119-Al. Molecular weight markers are indicated. Kpnl/EcoRl digested gtll-Al served as a positive control for the probe.


gt11-A1
Canton S
IIID
1 2 3 4 5 6 1 2 3 4 5 6
23.0
9.4
6.5
4.3
2.3
2.0
0.5

strain IIID also contained the Canton S fragment in addition to the one unique to itself (data not shown). Additional analysis of individual fly DNA samples was made from both the Canton S and strain IIID stocks used in this study and stocks maintained by other laboratory personnel. Southern analysis indicated strain IIID DNA obtained from other stocks contained the faster migrating EcoRl fragment only and that the extra fragment similar in mobility to that in Canton S must be due to contamination of the strain IIID stock used in this study with Canton S (data not shown). Double digestions of BamHl/HindIII, HindIII/EcoRl and BamHl/EcoRl resulted in similar results for the two strains. A restriction map of the genomic DNA in the vicinity of the probe, derived from the data in Figure 25 was made for Canton S (Figure 26). The position of the polymorphic EcoRl site in strain IIID is indicated by "E*".

3.6.2 Analysis of Total RNA

Total RNA from larval, pupal and adult stages of Canton S and strain IIID was analysed with hybridization to pUC119-neg and pUC119-Al probes and an actin probe which served as a control for the amount of RNA present. Dot blots containing RNA from the two strains at different stages were hybridized with the three probes (Figure 27). Varying amounts of RNA were applied with a range of .625 to 5 µg to attempt to titer out the probes. Probe pUC119-neg hybridized only to the control plasmid DNA applied and not to any

Figure 26: Genomic restriction map for the region hybridizing to the pUC119-A1 insert probe, derived from the Canton S data from the Southern in Figure 25. The location of the polymorphic EcoR1 site found in strain IIID DNA is indicated by E*. "E" EcoR1; "H" HindIII; "B" BamH1. The starred bar underneath the map indicates the region of DNA to which the probe bound.

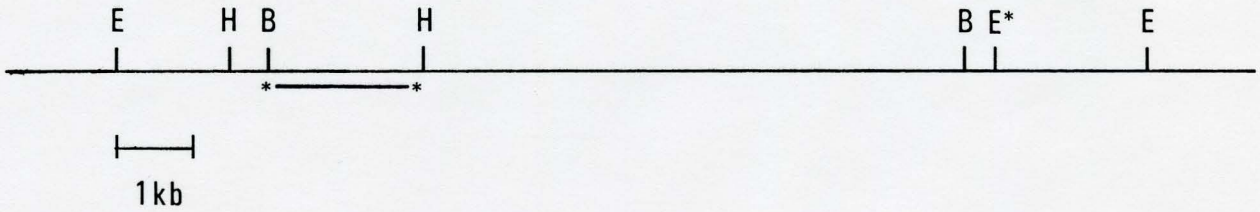
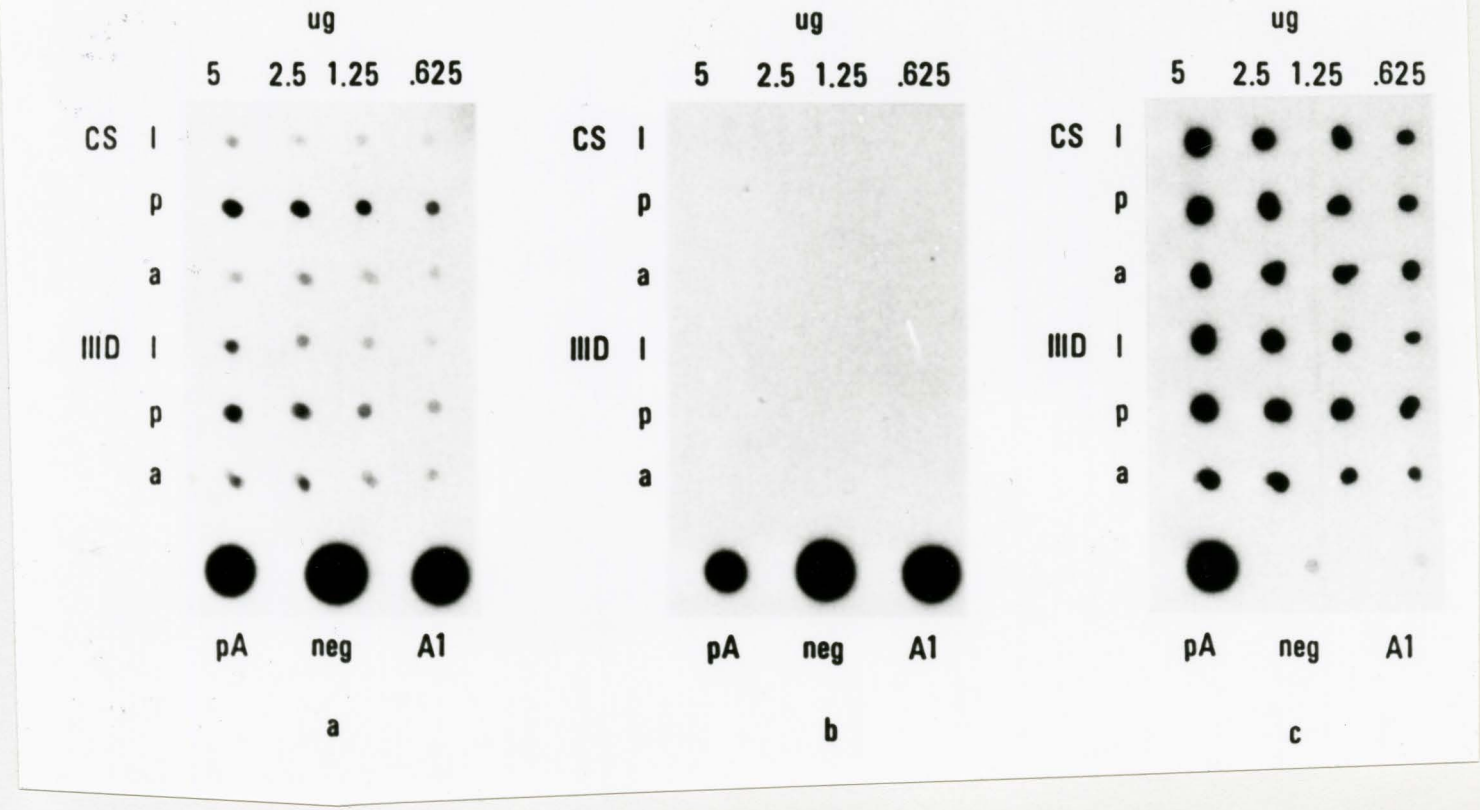

E
H
B
H
B
E*
E
1kb

Figure 27: Autoradiogram of total RNA dot blots from larval, pupal and adult stages of both Canton S and strain IIID, probed with (a) pUC119-A1 insert; (b) pUC119-neg insert or (c) pActin insert. The amount of RNA applied in each case is indicated. Control plasmid or phage DNA (pActin, gt11-neg and gt11-A1) was applied to act as controls for each probe.


ug
5
2.5
1.25
.625
CS
I
p
a
IIID
I
p
a
pA
neg
A1
a
b
c

RNA samples. Probe pUC119-A1 did hybridize to the RNA samples indicating its insert did contain coding cDNA. As expected the actin probe hybridized to all RNA samples. Probes pUC119-neg and pUC119-A1 hybridized to the pActin control plasmid DNA presumably because they were contaminated with pUC119 sequences. The pActin plasmid is also a pUC derivative plasmid. In addition to autoradiography, the dots were cut from the filters and counted in a scintillation counter to determine the precise amount of hybridization in each case (Table 3). Levels of probe A1 hybridization were divided by levels of probe actin hybridization to remove effects caused by variable amounts of RNA. The results are given in Table 4. More A1 probe hybridized to strain IIID RNA than to Canton S RNA in the larval and adult stages suggesting that perhaps more message was present in strain IIID, correlating with higher cytochrome P450 levels in strain IIID. In the pupal stage however, the data indicated that more A1 probe bound to Canton S RNA. Statistical analysis was used to determine if the amounts of hybridization of probe A1 in Canton S and strain IIID were significantly different. A t-test indicated that pupal and adult strain differences were significant ($P<0.05$) while larval probe A1 hybridization was not. The fact that earlier results (section 3.6.1) suggested that strain IIID DNA might be contaminated with Canton S DNA must be considered in interpreting these results.

Table 3: Average Counts of Probes Al and pActin Corrected for Background

		Canton S		IIID	
	µg	Al	pActin	Al	pActin
larval	5	41.5	753.0	462.9[1]	582.0[1]
	2.5	25.8	365.9	42.8	362.6
	1.25	40.6	224.5	24.8	183.0
	0.625	20.4	105.0	16.5	61.9
pupal	5	179.6	692.2	144.0	665.3
	2.5	147.7	405.7	98.9	430.5
	1.25	106.9	248.4	54.5	317.1
	0.625	59.3	136.6	41.2	163.9
adult	5[2]	43.9	282.6	69.3	233.5
	2.5	83.6	305.5	60.6	195.2
	1.25	54.6	188.0	37.2	99.6
	0.625	25.6	179.0	27.6	54.5

(1) These values were ignored for the analysis in Table 4 due to the exceedingly high value of Al hybridization.

(2) Less than 5 µg was applied to the blot.

Table 4: Hybridization of Al Probe Relative to Actin Probe

	Canton S	IIID	IIID/Canton S	t-test[1]
Larval	.125 (.036)	.173 (.047)	1.38	ns
Pupal	.372 (.041)	.217 (.017)	0.58	$P<0.05$
Adult	.216 (.039)	.371 (.048)	1.72	$P<0.05$

(1) t-test (two tailed), 6 degrees of freedom for difference between Canton S and IIID means.

Discussion

The intent of this study was twofold. Differences in cytochrome P450 between insecticide resistant and insecticide susceptible strains of Drosophila melanogaster were examined by SDS polyacrylamide gel electrophoresis and Western immunoanalysis. Secondly, using antibodies directed against proteins present in increased amounts in resistant strains an expression vector library was screened in an attempt to obtain a structural gene for cytochrome P450 from Drosophila melanogaster. Several clones were isolated and one was used in the analysis of genomic DNA and total RNA from both resistant and susceptible strains.

4.1 Analysis of Cytochrome P450 in Drosophila melanogaster

Cytochrome P450 in Canton S, an insecticide susceptible strain, and the resistant strain IIID was examined. Total cytochrome P450 contents indicated that strain IIID had levels five times higher than that of Canton S (Table 1 and Figure 6). This increase could have been due to the presence of a few new forms of cytochrome P450 or to an overproduction of some or all existing forms. Upon SDS polyacrylamide gel electrophoresis and silver staining, strain IIID and other resistant third chromosome strains were observed to contain two proteins that were greatly enhanced in quantity (Figure 1). Canton S also appeared to

melanogaster. One protein, designated P450a, of size 59 kd, was ubiquitous to both resistant and susceptible strains. A second protein, designated P450b, of size 56 kd, was expressed only in those strains resistant to insecticide. The question arises as to whether the 56 kd protein identified by Waters as P450b is the same as the protein designated P2 in this study. Any differences in molecular weight could be attributed to different methods of analysis used in the two laboratories. When microsomal proteins from chromosome III strains isolated by this laboratory were analyzed alongside microsomal proteins from resistant lines isolated by Waters the P450b protein (56 kd) appeared to migrate to an intermediate position beteen P2 and P3 (Sundseth, personal communication). P2 was definitely larger than P450b although independently determined molecular weights suggested the opposite. Naquira et al (1980) reported three cytochrome P450 species in Drosophila melanogaster, designated $P\text{-}450_{Dr.449}$, $P\text{-}450_{Dr.451}$ and $P\text{-}450_{Dr.450}$ with molecular weights of 51.75 kd, 50.8 kd and 54.8 kd respectively when examined by SDS-PAGE. Again it is possible that $P\text{-}450_{Dr.450}$ is the same as P2 and $P\text{-}450_{Dr.449}$ or $P\text{-}450_{Dr.450}$ is the same as P3. From the work done in various laboratories it can be assumed that at least five cytochrome P450 isozymes exist in Drosophila melanogaster. It would be interesting to compare P2 and P3 with the cytochrome P450s identified by Hällström and Naquira to see if they are the same.

Polyclonal antibodies directed against CHAPS solubilized column purified strain IIID microsomal proteins have proved useful in characterizing P2 and P3. The antibodies Abl-B, directed against P2, and Ab2-IgG, directed against P2 and P3, were both also reactive to a lesser degree with other microsomal proteins (Figure 8). When immunostained with either of these antibodies, strain IIID contained much higher levels of P2 and P3 than Canton S (Figure 8) confirming the result obtained by silver staining that these proteins were enhanced in chromosome III resistant lines. The results of two separate experiments using these antibodies suggested they were directed towards cytochrome P450 species. Ab2-IgG added to strain IIID microsomal samples decreased the total amount of cytochrome P450 as measured by CO difference spectra, to 53% of the original (Figure 9). Not all cytochrome P450 could be removed after extended incubation possibly due to insufficient amounts of antibody or the presence of some cytochrome P450 isozymes not antigenic towards the antibody. Antibody/antigen complexes pelleted from the samples indicated the presence of P2 and P3 (Figures 10 and 11). A second experiment showed there was a positive correlation between the antigenic response of P2 and mixed function oxidase activity in a variety of lines. Lines with low mfo activity tended to have little P2, as determined by immunoanalysis with Abl-B, while lines with higher mfo activities had increasing amounts of P2 (Figure

12). Other proteins also immunogenic to Abl-B showed no correlation between antigenic response and mfo activity. There are two conclusions to be made from the results of these experiments. Proteins P2 and P3 are almost certainly isozymes of cytochrome P450 in Drosophila melanogaster. Secondly, the antibodies produced through injection of microsomal proteins from the resistant strain into a rabbit, although polyclonal, are largely directed to P2 and P3.

4.2 Characterization of a Lambda gtll Clone Antigenic to Ab2-IgG

Ab2-IgG, antigenic to both P2 and P3, both putative cytochrome P450s, was used to screen a lambda gtll library. The lambda gtll library consisted of cDNA fragments obtained from a lambda gtlO library and ligated into lambda gtll arms. Forty-three thousand insert-containing clones were screened. Assuming 5000 genes are present in the Drosophila genome it is unlikely that all genes were expressed as fusion proteins in this library. The lambda gtlO library was amplified prior to ligation with the lambda gtll arms, hence all represented clones were probably present more than once. In addition, because this screening method is dependent on genes being inserted into the vector in the correct orientation and in the correct reading frame, only one out of every six clones produces an antigenic fusion protein. In spite of this three clones were determined to be antigenic towards the antibody, of which one, designated

gtll-Al, was very immunogenic. On examining stained filters both negative and positive plaques could be visualized (Figure 14). Negative plaques tended to have a donut shaped appearance probably due to the deposition of E. coli proteins from recently lysed cells that subsequently reacted with anti E. coli antibodies in the polyclonal rabbit serum. In contrast positive plaques were a distinctly solid purple color. The screening procedure is such that protein production is induced from the β-galactosidase promotor in all clones. In non insert-containing clones this results in the overproduction of β-galactosidase to which there were no antibodies in the serum used. In insert-containing clones a fusion protein is produced. Should this protein have antigenicity towards the serum used, the large amount of antigenic protein present results in a solid, dark purple plaque. Although several sources had suggested a high rate of background due to anti E. coli antibodies in the polyclonal rabbit serum (Huynh et al, 1986; Snyder et al, 1987) this did not appear to be a problem when using alkaline phosphatase-linked second antibodies for detection. The problem of cross hybridization with E. coli proteins is probably enhanced when using isotopic means of detection. Autoradiography tends to result in slightly fuzzy images which would make it difficult to tell the difference between positive and negative plaques. Colourimetric detection systems however, lend themselves to very distinct images.

Clone gtll-Al produced a fusion protein of approximately 130 kd that was immunogenic towards both Ab2-IgG and an anti β-galactosidase antibody. This fusion protein contained 114 kd of β-galactosidase, leaving 16 kd coded for by the insert in the clone. The fact that gtll-Al was very immunogenic towards Ab2-IgG coupled with the fact that Ab2-IgG was immunogenic largely towards P2 and P3 suggests that it may code for one of P2 or P3. One very important experiment yet to be done is to confirm that gtll-Al is a clone for P2 or P3 and that the sequences it contains are part of a Drosophila cytochrome P450 gene. This could be accomplished by sequencing the insert in gtll-Al and comparing it to other cytochrome P450 sequences. Another mutually exclusive approach would be to show that antibody cross reactivity existed between the gtll-Al fusion protein and either P2 or P3. This could be accomplished using a double diffusion test, a method by which cross reactivity can be observed. The fact that the clone only coded for a 16 kd protein can be interpreted in a number of ways. It is possible the clone does not code for cytochrome P450 but instead codes for a 16 kd unrelated protein that was antigenic to Ab2-IgG. When whole fly homogenates are immunostained with antiserum Abl-B however, no bands in this size range exhibit intense staining (Nicholas Okoampah, personal communication). In addition one would expect the antibody staining gtll-Al to be a major component of Ab2-IgG, as the level of response in gtll-Al

was so high. Only antibodies against P2 or P3 meet this requirement (Figure 8). A more likely explanation would be that gt11-A1 does code for cytochrome P450 but the full coding sequence is not represented in the clone. It is possible some cDNAs inserted into the library were not full length due to incomplete or degraded message. In addition when creating the lambda gt10 library from which the lambda gt11 library was made, EcoR1 sites were not well methylated. As a result when linkers were removed by EcoR1 digestion, internal sites were also cut resulting in shorter than full length inserts. Shorter inserts can be advantageous because the resulting smaller fusion proteins are generally more stable than larger fusion proteins.

Very few clones were isolated in the initial screen, in fact only gt11-A1 was highly antigenic. The fact that more were not found could be explained by improper reading frames or orientation of inserts. In addition immunoanalysis (Figure 17b) indicated the fusion protein was not very stable and broke down to β-galactosidase and smaller fragments. Hence if a clone produced a very unstable fusion protein it would probably not be detected by antigenic screening. If the lambda gt11 library was rescreened using the gt11-A1 insert as a nucleic acid probe most likely several more clones would be picked up. A second possible explanation why so few clones were isolated could be that the antibody used to screen the library may only be antigenic towards

certain epitopes of the cytochrome P450 molecule. There may be more clones present in the library producing partial cytochrome P450 fusion proteins which do not express appropriate epitopes.

As described in the results section (3.5.2) the EcoRl site closest to the left end of the gtll-Al vector was apparently destroyed during the cloning process. A cDNA insert of .5 kb was successfully isolated as part of a 1.5 kb Kpnl/EcoRl fragment and was subcloned into pUCll9 (Figure 22). Because this clone contained lambda gtll DNA it was necessary to also subclone the 1 kb Kpnl/EcoRl fragment from gtll-neg (a non insert-containing lambda gtll clone) to serve as a control. Southern analysis was used to determine if the gtll-Al insert had any homology to other potential cytochrome P450 clones isolated by this laboratory or by other laboratories. Even at low stringency the Al insert did not hybridize to any potential cytochrome P450 clones, including A2 and Cl. This latter fact was not surprising as the antigenic responses between Al and A2 and Cl were so different. Inserts from other independently isolated putative cytochrome P450 clones (pDMP450, gtll-5111A and gtll-BC) also did not hybridize to the pUCll9-Al insert. Assuming any of these clones did code for cytochrome P450 it is conceivable that they do not hybridize to the pUCll9-Al insert because they contain coding sequences for different portions of the gene.

4.3 Analysis of Genomic DNA with the gt11-A1 Insert

Genomic DNA from Canton S and strain IIID was analysed by Southern hybridization with the pUC119-A1 insert probe. Initially it was necessary to determine that the gt11 portion of the probe would not bind to Drosophila genomic DNA. By dot blot analysis it was shown that the pUC119-A1 probe only, bound to genomic DNA at the high stringency conditions used ($68^{o}C$) (Figure 24). Southern analysis of genomic DNA revealed a polymorphism between Canton S and strain IIID when digested with the enzyme EcoR1 (Figure 25), although other enzymes used (BamH1 and HindIII) resulted in apparently identical patterns in the two strains. Strain IIID contained a smaller EcoR1 fragment than did Canton S. On some occasions however, strain IIID also contained a fragment equal in size to that of Canton S. It is believed this fragment was due to contamination of the stock culture with Canton S and that the true strain IIID fragment was the smaller fragment. Although this polymorphism may not be involved in the sequence of the gene of interest, it could be very useful in determining the genomic location of the DNA fragment being examined. By way of genetic crosses it would be possible to determine with which chromosome the polymorphism was associated. From the restriction data obtained from Southern analysis it was possible to construct a partial genetic map for the region of DNA to which the probe bound (Figure 26).

4.4 Analysis of Total RNA

Total RNA from various stages of Canton S and strain IIID was analysed by hybridization to the pUC119-A1 insert probe. To ensure that the lambda gt11 sequences were not hybridizing to Drosophila RNA the pUC119-neg insert probe was used as a control. It did not bind to any RNA from either strain (Figure 27b). In addition an actin probe was used to serve as a control for the amount of RNA present on the filter (Figure 27c). The pUC119-A1 insert probe did hybridize to the RNA indicating it did contain coding sequences (Figure 27a).

Dot blots were analysed by cutting out each spot and measuring their radioactivity on a scintillation counter to obtain total counts (Table 3). To remove the effect of RNA amount, levels of A1 hybridization were divided by levels of pActin hybridization. The results are shown in Table 4. Assuming the gt11-A1 insert does code for a structural gene for cytochrome P450 it was expected strain IIID would contain more hybridizing message than Canton S as higher levels of cytochrome P450 are found in strain IIID. In the larval and adult stages strain IIID did have higher levels of A1 hybridization. A two-tailed t-test indicated however these differences were significant at a 95% confidence level in the adult stage only. Cytochrome P450 and mfo activity are highest in larval and adult stages and almost negligible in the pupal stage. Assuming that gt11-A1 were a clone for

cytochrome P450, one would expect the smallest amount of message to be present in pupal RNA. This however was not observed. Table 4 indicates that both Canton S and strain IIID exhibited a substantial amount of signal in the pupal RNA. In addition, Canton S pupal RNA had a level of Al hybridization that was higher than that of strain IIID pupal RNA. This difference was significant at a 95% confidence level. The reason for the abundance of message in the pupal RNA is confusing. It is possible nonspecific binding of the probe is affecting these results. Northern analysis would be required to resolve these discrepancies.

RNA was spotted onto the filters in a dilution series with the intent of titering out pUC119-Al hybridization allowing comparisons to be made between various strains or stages. One problem encountered was that the amount of hybridization was not proportional to the amount of RNA present when probed with the pUC119-Al insert probe (Table 3). The actin probe, which had relatively high levels of hybridization, did show a good correlation to the amount of RNA present. The pUC119-Al insert probe however, which had much lower levels of hybridization, did not show proportional increases with the addition of more RNA. It appears insufficient Al hybridizing RNA was present to obtain a good signal. This contributed to the failure to obtain definitive results.

The RNA results are very preliminarly but initially it does appear that strain IIID has more mRNA homologous to the pUC119-A1 insert than does Canton S in the larval and adult stages. The pupal stage does present some confusing results that have yet to be resolved.

4.5 Evidence for Regulatory vs Structural Loci controlling Cytochrome P450 Expression

Increases in cytochrome P450 observed in resistant strains of Drosophila melanogaster can be the result of changes in structural loci or regulatory loci. A structurally new cytochrome P450 may have a more accessible heme group resulting in apparently more cytochrome P450 or higher activities towards substrates resulting in greater resistance. Regulatory changes could result in the overproduction of existing forms of cytochrome P450 already involved in detoxification processes. Studies have suggested regulatory genes play a large role in insecticide resistance. Plapp and Wang (1982) studied insecticide resistance in the housefly. They examined inversions observed in resistant strains that resulted in bringing important genes closer to each other. From studies of these strains they concluded a regulatory gene was important for increased levels of cytochrome P450 in resistant strains. Regulatory genes are known to play roles in some enzyme systems in Drosophila melanogaster. Production of the alcohol dehydrogenase enzyme in Drosophila is controlled by a regulatory

gene on the third chromosome although the structural locus is located on the second chromosome (Ayala and McDonald, 1980). This regulatory gene controls the amount of enzyme present apparently by directing a regulatory unit to bind at a control site adjacent to the structural gene locus.

The results of this study indicate that in Drosophila there is an overproduction of a few specific existing forms of cytochrome P450. This suggests the presence of a regulatory gene controlling structural gene expression. Hällström et al (1984) found certain cytochrome P450s were associated with chromosome II. Strain IIID, examined in this study, is a strain isochromosomal for a resistant third chromosome. This discrepancy could be resolved if chromosome III contained a regulatory gene that controlled cytochrome P450 expression from a gene elsewhere in the genome, possibly on chromosome II. Waters and Nix (1988) suggested the structural gene for P450b in Drosophila was located on the second chromosome however a resistant third chromosome was required for its maximum expression. Two regulatory loci near hairy (3-26.5) and curled (3-50.0) were identified as being important. There were also some minor positive effects when resistant first and fourth chromosomes were present. Houpt et al (1988) described some resistant chromosome II strains obtained form the same original population as strain IIID that did not show correlations between increases in protein content and cytochrome

P450. This further supports the notion that chromosome III is required for maximum cytochrome P450 expression. Indications from this study and others suggest in Drosophila insecticide resistance is the result of an increase in cytochrome P450 due to the effect of a regulatory gene probably located on the third chromosome.

4.6 Future Research

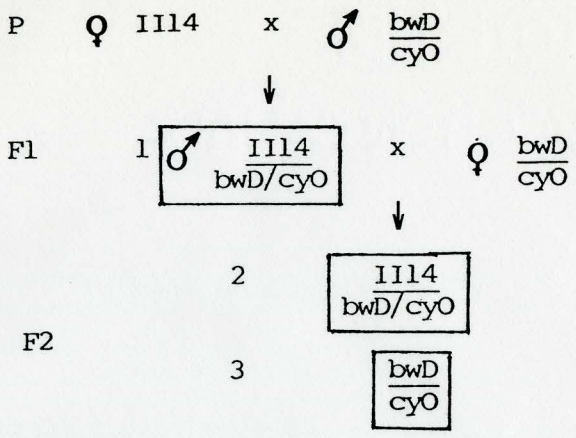
This study has made an initial attempt at identifying a structural gene for cytochrome P450 in Drosophila melanogaster. It remains to be confirmed that the clone gt11-A1 contains cytochrome P450 sequences. Once a confirmation of its identity as a cytochrome P450 coding sequence is made, more and full length clones could be isolated from the library by using the gt11-A1 insert as a nucleic acid probe. In addition the problems with RNA analysis need to be resolved. Future research could include determining the chromosomal location of the gene by in situ hybridization to salivary gland chromosomes. The resistance factor could be transferred to susceptible strains using P-element mediated transposition. The isolation of a gene for cytochrome P450 from a well defined insect species such as Drosophila melanogaster would be very useful as a probe for investigating insecticide resistance in other more damaging insect pests.

Appendix A

The putative cytochrome P450 clone, pDMP450, was a gift of Dr. B. Cochrane, Department of Biology, University of South Florida. It contains a 2.1 kb insert that was subcloned from a lambda clone of the Maniatis Drosophila genomic library. The lambda clone showed weak homology to a mouse phenobarbitol induced cytochrome P450 gene. The pDMP450 insert was used as a probe in Southern analysis of restricted DNA from Canton S, strain II14 (a malathion resistant strain isochromosomal for a second chromosome (Halpern and Morton, 1987)) and strain IIID. Upon analysis, a HindIII polymorphism amoung all three strains was observed (Figure 29). Other enzymes used (EcoR1 and BamH1) indicated no polymorphisms (data not shown). The HindIII polymorphism was used as a marker to determine which chromosome the pDMP450 insert was binding to. By making genetic crosses and using Southern analysis it was possible to follow the polymorphism through the crosses. Two chromosome balancer strains (strains containing inversions on one of the chromosomes which prevent crossing over) were used. A balancer for the second chromosome was bw^{D}/cyO characterized by brown eyes and curly wings. The balancer for the third chromosome was MKRS/TM3 characterized by stubble and serated wings. Crosses were made as described in Figure 28. DNA preparations were made from the populations indicated. Table 5 indicates the expected

Figure 28: Diagram of the three genetic crosses made to determine the location of the polymorphic HindIII site. Populations that were analysed for the polymorphism are boxed and numbered.

CROSS I:



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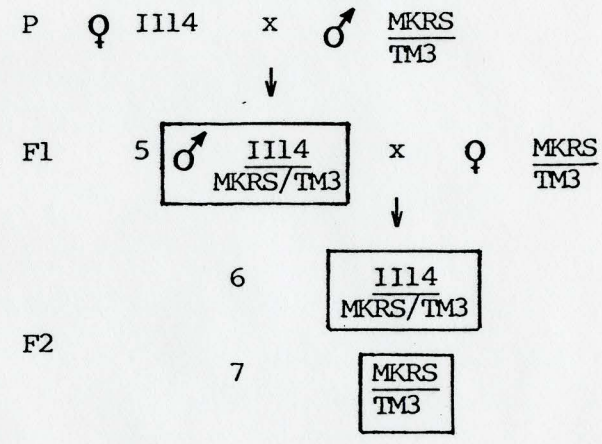


CROSS III:

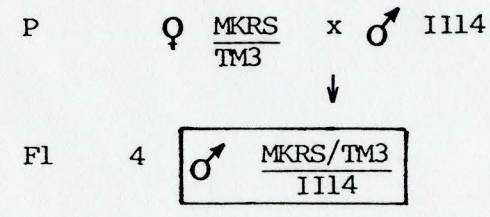


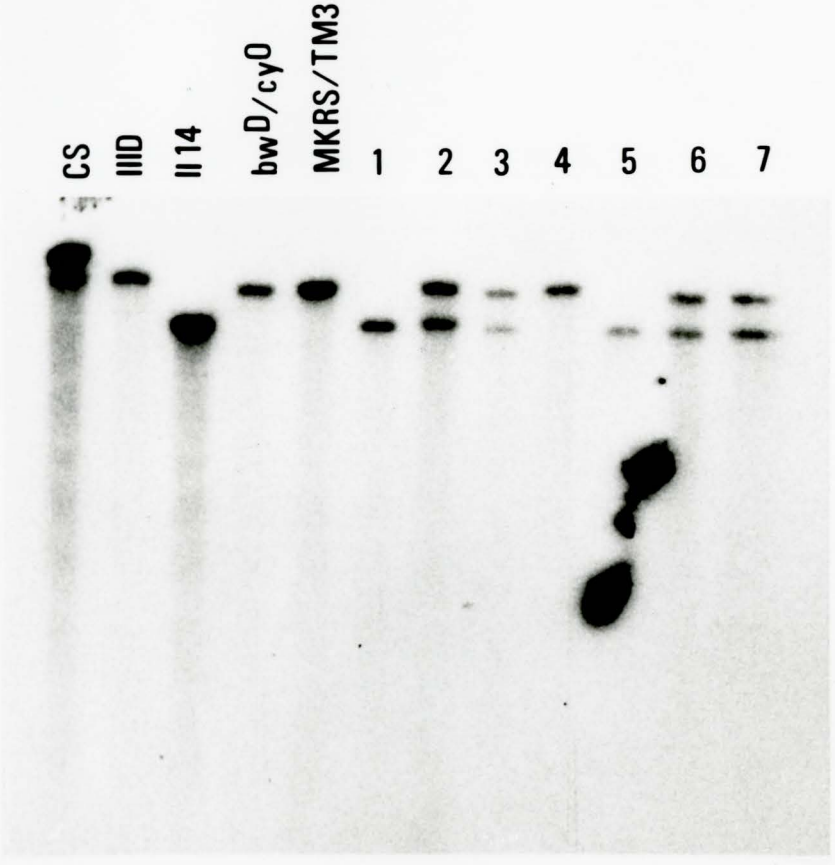
Table 5: Expected Restriction Patterns for the HindIII Polymorphism

Fly type	location of polymorphism		
	X	CII	CIII
II14	|	|	|
bw^D/cy^O	|	|	|
MKRS/TM3	|	|	|
II14/bw^D/cy^O F1	| |	| |	| |
II14/bw^D/cy^O F2	| | *	| |	| |
bw^D/cy^O F2	| | *	|	| |
MKRS/TM3/II14 F1	|	| |	| |
II14/MKRS/TM3 F1	| |	| |	| |
II14/MKRS/TM3 F2	| | *	| |	| |
MKRS/TM3 F2	| | *	| |	|

* In these cases the II14 fragment is only present in the female F2 however because flies were not sexed for the purpose of F2 DNA preparations both fragments are seen.

HindIII restriction patterns for each fly type given the polymorphism was on chromosome I, chromosome II or chromosome III. Southern analysis was carried out on each fly type and probed with the pDMP450 insert (Figure 29). The results indicate conclusively that the polymorphism is associated with the first (X) chromosome. Although previous studies have not linked cytochrome P450 and associated activities to the X chromosome it is possible that structural genes are located here and are controlled from genes elsewhere in the genome.

Figure 29: Autoradiogram of a Southern blot hybridized to the pDMP450 insert probe. DNA samples were digested with HindIII. Lanes 1 through 7 refer to populations indicated in Figure 28. Lane 1 II14/bw^D/cyO male F1; lane 2 II14/bw^D/cyO progeny; lane 3 bw^D/cyO progeny; lane 4 MKRS/TM3/II14 male F1; lane 5 II14/MKRS/TM3 male F1; lane 6 II14/MKRS/TM3 progeny; lane 7 MKRS/TM3 progeny.


CS
IIID
II 14
bwD/cyO
MKRS/TM3
1
2
3
4
5
6
7

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