Investigation of Bax VDAC Interactions and Their Relationship Regarding Apoptosis in Drosophila melanogaster

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INVESTIGATION OF BAX VDAC INTERACTIONS AND THEIR RELATIONSHIP REGARDING APOPTOSIS IN DROSOPHILA MELANOGASTER

by

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

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ABSTRACT
JOHN MAGRUDER SULLIVAN III: Investigation of Bax VDAC Interactions and Their Relationship Regarding Apoptosis in *Drosophila melanogaster* (Under the direction of Dr. Bradley W. Jones)

Cell mediated death, or apoptosis, is a critical biological process that once fully understood could unlock a potentially new understanding of the mechanisms of both cancer and neurodegenerative diseases. The general mechanism of apoptosis includes cytochrome c being released from the mitochondrial membrane through a channel created by an activated pro-apoptotic BH123 protein. Once the cytochrome c leaves the mitochondrial membrane it goes on to ultimately activate a caspase cascade, which results in cell apoptosis. Similar to BH123 channels, VDACs (voltage-dependent anion channels) are also pore-forming proteins that regulate the intake and output of metabolites from the mitochondrial intermembrane space and cytosol. Dr. Jekabsons has shown in rat granule neurons that the BH123 protein *Bax* interacts with VDACs. He was able to show that when a *Bax* VDAC complex forms, *Bax* is not active. From that, a hypothesis was formed to test whether VDACs inhibit the activation of *Bax* and therefore restrain apoptosis.

To test this, we used *Drosophila melanogaster* as a genetic platform test the hypothesis that VDAC prevents premature *Bax* activation by crossing a VDAC mutant with one that ectopically expresses *Bax*. The extent of *Bax*-mediated death in this fly
strain was compared to a strain functional VDAC and \textit{Bax} alleles. If the hypothesis was true, the crosses were expected to show that the strain with an inactive VDAC and an active \textit{Bax} would have a worse visible phenotype due to increased cell death attributed to less restraint from the VDACs on apoptosis. The Gal4 UAS system was used to induce selective activation of the \textit{UAS-Bax} gene in the eye of \textit{Drosophila melanogaster} by using the \textit{GMR-Gal4} to selectively activate it.

The VDAC mutant that was used was \textit{porin}^{365}, which contains a deletion and therefore inactivates the gene. This \textit{porin} mutant was crossed through many generations to form a final genotype of \textit{porin}^{365} \textit{GMR-Gal4/UAS-Bax}. The \textit{porin}^{36} \textit{GMR-Gal4} chromosome was from a recombinant line that was created during the early stages of the genetic crossings.

The final results indicated that the eye phenotype in the flies with an activated \textit{Bax} gene and an inactive \textit{porin} gene had less apoptosis than flies expressing functional \textit{porin}. Increased cell death in the eye did not occur and therefore we were not able to lend support for the hypothesis that VDACs negatively impact the function of \textit{Bax} in apoptosis. In fact the results tend to indicate a possibility that VDAC may have a role in facilitating \textit{Bax}-dependent apoptosis.
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Introduction

Apoptotic Outline

Cell mediated apoptosis is an important mechanism for removing excess or dysfunctional cells that is not fully understood. For various reasons, cells can initiate apoptosis, which in turn causes the cell to disassemble and collapse prior to being taken up by another cell. After initiation of apoptosis, procaspases, which are inactive proteins initially, are cleaved into active caspases. Caspases are proteins that contain an active site with a cysteine amino acid and that cleave proteins at their specific aspartic acid sites. Once the activation of the caspases begins a cascade forms allowing for the activation of more caspases from the initially active caspase. Once active, the caspases activate other executioner procaspases, which in turn work to break down the various components within the cell. (Alberts, 2002).

The initiation of apoptosis begins with the release of cytochrome c from the mitochondria into the cytosol of the cell. This release ultimately activates apoptotic protease activating factor-1 (Apaf1) in the cytosol and through ATP hydrolysis forms a multi-Apaf1 circular structure known as an apoptosome. The apoptosome then activates the procaspases after they have joined the apoptosome. This super complex can further
activate procaspases such as executioner procaspases to initiate the steps that are outlined above. The figure below illustrates the activation of the apoptosome. (Alberts, 2002).

![Figure 1: Apoptosome Formation](Illustration from Alberts, 2002)

In order for cytochrome c to be released from the mitochondria, there must first be a BH123 pro-apoptotic protein activated to form a channel through which it can leave the mitochondria. In a normal cell, the BH123 proteins are inactive while the Bcl2 proteins are active. The Bcl2 proteins are active proteins on the mitochondrial membrane that prevent the release of certain mitochondrial proteins, which will activate apoptosis. When an apoptotic signal occurs, BH3 proteins, which make up a large portion of the Bcl2 proteins, are thought to activate and inhibit the anti-apoptotic Bcl2 proteins by binding to them in the mitochondrial membrane. This binding is thought to help activate the BH123 proteins that inactively reside in the mitochondrial membrane. Bax and Bak are the
primary BH123 proteins that propagate the initiation of apoptosis by allowing cytochrome c release from the mitochondria. (Alberts, 2002).

VDACs, also known as voltage-dependent anion channels, are pore-forming proteins that regulate the intake and output of metabolites from the mitochondrial matrix and cytosol. Although similar to the BH123 protein channels, it has been hypothesized that these channels negatively affect the activation of apoptosis by either Bax or Bak, as VDAC has been shown to interact with Bax in healthy cells (Huckabee and Jekabsons 2011). Potentially, the VDACs hold onto the inactive version of the BH123 proteins and prevent them from forming channels that allow for cytochrome c to leave the mitochondria and therefore activate apoptosis. A better understanding this relationship, therefore, is the basis for this research and is a product of research conducted by Dr. Mika Jekabons.

**Understanding the VDAC BH123 Relationship**

In order to further understand the functional relationship between VDACs and BH123 proteins, it is important to chose a model system in which the sense can be easily manipulated and the consequences of their interaction readily identified. *Drosophila melanogaster* is a good genetic platform to test this relation because it contains a quick life cycle. The VDAC homolog that is expressed in the *Drosophila melanogaster* is known as porin. In an active form, porin functions as a channel that allows various small metabolites such as ATP to cross the mitochondrial outer membrane. The BH123 protein that will be used is the Bax gene from a mouse. (Park, 2010). Bax was selected over its
homolog Debel that is ordinarily found in Drosophila because our hypothesis is based on Dr. Mika Jekabsons’ work with Bax in Rats. (Galindo, 2009).

The idea in testing this relationship relies on the ability to selectively activate Bax in a specific portion of the Drosophila melanogaster. Without a specific isolated activation, the test would not be possible due to the excessive cell death by the activated Bax protein.

By using the Gal4 UAS driver system it is possible to selectively express Bax in a specific tissue of the fly, namely the eye. By using the GMR-Gal4 driver for this system, it is possible to localize the cell death in only the eye because the GMR promotor selectively drives gene expression in this tissue. Through this system, the gene attached to the UAS promoter is only transcribed in the presence of the Gal4 gene and thus selective transcription is achieved. The activation of this ordinarily inactive gene will not impact the research because the GMR-Gal4 gene has no significant function on the Drosophila melanogaster eye. (Li 2012).

Another important aspect of this research is to work with a fly line that contains an inactive form of porin. The confirmation of an inactive form would be one that possesses a Polymerase Chain Reaction (PCR) generated DNA fragment that would show a band that proceeds further down the gel than the active version indicating that portions of the porin gene have been deleted and therefore are inactive. Figure 2 shows the possible porin mutants that could have been used for the cross. The three porin mutants contain a deletion that results in a shorter, inactive gene product. The lengths of the deletions can be visually compared to that of the wild type porin gene, which contains no deletions at the top of the figure.
Figure 2: *porin* Wild Type Versus *porin* Deletions
Illustration from Graham, 2010

Once the *porin* mutant has been confirmed, it can be recombined with a *GMR-Gal4* line so that *porin* and *GMR-Gal4* are maintained on the same chromosome. Once recombination occurs, the line can be crossed with *UAS-Bax* to create a fly line that contains both an inactive version of *porin* with an active form of *Bax* that is localized to the eye.

The hypothesis that is being tested is based off previous research that shows a *Bax VDAC* interaction in rat granule neurons (Huckabee and Jekabsons, 2011). If the hypothesis that VDACs negatively interferes with the activation of BH123 proteins is true then the recombinant flies expressing mutant *porin* and *Bax* should exhibit an increase in cell death. This cell death should be noticeable in the formation of the eye such that it creates a “rough” eye. The “rough” eye is simply a loss of order in the formation of the eye.
Figure 3: Rough Eye Phenotype Attributed to UAS-BAX Activation  
Picture from Gaumer, 2000

Figure 3 shows the abnormalities in the eye of the fly with an activated copy of Bax in the bottom two pictures compared to the wild type eye in the picture above. When the pictures are compared, the eye with activated Bax clearly lacks the symmetric organization of the wild type eye and this is due to the cell death. Parts C and D illustrate what is meant by the “rough” eye phenotype.

For the current experiments, there are three possible phenotypic outcomes resulting from the crosses. First, the “rough” eye phenotype with mutant porin could be more severe than that of the GMR-Gal4 UAS-Bax flies with wild type porin. Such a result would be consistent with porin functioning as an inhibitor of Bax, as less would be sequestered by reduced porin expression. Second, if the phenotype of the eye is not
affected in the *porin* mutant then the interaction of mammalian *Bax* with *Drosophila porin* may not occur, possibly due to sequence differences between *Drosophila* and mammalian *porins*. Third, if the deletion of *porin* improves the eye phenotype then this would be consistent with *porin* functioning to facilitate *Bax* activation. If this is the case then by removing a copy of *porin* the apoptotic affect exhibited by *Bax* should be further limited than in that of the original *GMR-Gal4/UAS-Bax* flies.

If the data are consistent with the hypothesis such that deleting once copy of *porin* worsens the eye phenotype, then further tests could be done with other strains to confirm the interaction. Additionally, deleting both copies of *porin* is predicted to result the eye having a more sever phenotype than that of the single *porin* deletion.

It is my hope that through a successful recombination genetic experiment that I will be able to distinguish recombinant lines that contain a *GMR-Gal4* and *porin* chromosome via PCR, and thus be able to note a worsening of the “rough” eye phenotype. The worsening of the eye should therefore be attributed to the selective expression of *Bax* and potentially help further explain its role in apoptosis based on its relation with VDACs.
**Methods and Materials**

*Genetically Formulated Fly Strains*

Initially the *porin*\textsuperscript{78} mutant line of *Drosophila melanogaster* was used, but after doing an initial PCR to confirm the mutation, it was discovered that the *porin* gene was identical to that of the wild type flies. Instead, *porin*\textsuperscript{365} mutants were selected to begin the genetic crossing. Males from this strain that contained a genotype of *porin*\textsuperscript{365}/SM6B were crossed with virgin females with *GMR-Gal4/CyO*. Because *CyO*, or *Curly of Oster*, is a visual balancer, it was possible to eliminate the offspring with curly wings and therefore only select the offspring that contained the *porin*\textsuperscript{365}/*GMR-Gal4* genotype. Some of the *porin*\textsuperscript{365}/*GMR-Gal4* flies were tested via a fly prep and PCR to indicate they were in fact the correct genotype before proceeding. Females from the *porin*\textsuperscript{365}/*GMR-Gal4* strain were then crossed with *Scutiod (Sco)* over *CyO* as a visual indicator for progeny flies. 54 vials of potential recombinant flies were created and allowed to grow for a few generations. The hope is that some of these progeny contain a recombinant chromosome with *porin*\textsuperscript{365} and *GMR-Gal4* and another chromosome with either *Sco* or *CyO* as a visual balancer.

*Testing Procedures*
Fly Preparations

Following the appropriate genetic crossings the flies were tested to ensure the appropriate genotype had been obtained. The optimal genotype for in the porin mutant flies was one that contained both the porin mutant as well as the Gal4 gene. From the 54 vials, the first 24 were selected for analysis of genomic DNA by PCR. The flies that were chosen either contained the Sco phenotype (lack of bristles) or the CyO phenotype (curly wings) but not both. The DNA preparations began by squishing 2 randomly selected flies from each of the 1.5mL microcentrifuge tubes with a pipet tip while dispensing 50 µl of squishing buffer. After completing the squishing process, 1 µl of Proteinase K was added and the solution was incubated at 37°C for 25 minutes. These steps were intended to both mechanically and chemically open up the individual cells of the fly and release DNA into the solution. The samples were then placed on a 95°C heating element in order to denature the Proteinase K and the fly proteins.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used to amplify the DNA that was in solution to try to locate the potential recombinant lines. A master mix of PCR reactants was used that contained 324 µl of De-ionized water, 60 µl of dNTPs, 60 µl of PCR Buffer, 60 µl of Porin Forward2 Primer, 60 µl of Porin Reverse Primer, 60 µl of Gal4 Forward Primer, 60 µl of Gal4 Reverse Primer. Each PCR tube received 28.5 µl of the master mix along with 0.5 µl of Taq Polymerase and 1 µl of the particular sample it was assigned. The primers were designed by Dr. Jones to flank the porin deletion and to amplify the Gal4 gene. Following the PCR, the samples (5 µl each DNA prep plus 3 µl of
water and 1 µl loading dye) were run on a 1% agarose gel in 1X TAE at 90 volts. A DNA ladder was prepared by combining 1 µl of 10 kb ladder with 8 µl of water and 1 µl of loading dye.

From this initial PCR, 7 potential recombinants were selected from the sample of 24. The 7 samples were pulled and another PCR was performed to ensure that they were recombinants. As well as using the same fly preps from before, a new set of fly preps was also created to double check the work. From those 7 samples, 5 were selected as lines that contained both the $\text{porin}^{365}$ allele and $\text{GMR-Gal4}$ recombination.

The newly selected 5 lines of potential recombinants were isolated and grown for a few generations. From these lines, straight wing virgins were selected and crossed with males that had the genotype of $\text{UAS-Bax/TM6Sb}$. The straight winged flies were chosen because their genotype should be two copies of the $\text{porin}^{365}$ and $\text{GMR-Gal4}$ chromosome with the $\text{CyO}$ chromosome no longer a part of the genotype. The flies were crossed at 25°C to maximize the effectiveness of the UAS-Gal4 system. (Klueg, 2002).
Results

Creating the Drosophila Recombinants

Before testing the *porin Bax* relationship, a fly line that contained a potential recombinant chromosome consisting of both a *porin* mutant gene as well as a *GMR-Gal4* gene had to be created. Based on the *porin* mutants tested by Graham et. al. (Graham, 2010), the initial *porin* mutant strain that was selected was *porin*\textsuperscript{78}. Unfortunately, after beginning the genetic crossing, it was discovered through PCR that the *porin*\textsuperscript{78} strain actually contained an intact *porin* gene. (Figure 4)

![Figure 4: PCR of *porin* Mutants](image)
Lane 1 is a 10kb ladder, Lane 2 is the porin\textsuperscript{27} strain with an intact porin gene, Lane 3 is vial one of the porin\textsuperscript{78} mutants, Lane 4 is vial two of the porin\textsuperscript{78} mutants, Lane 5 is the porin\textsuperscript{365} mutants, and Lane 6 is the GMR-Gal4 line.

The gel shows that the supposed porin\textsuperscript{78} has the same size PCR product as that of porin\textsuperscript{27}, which contains an intact porin gene. Because the gene is seemingly intact, the porin\textsuperscript{78} strains were discarded and a different porin allele was selected and used in our experiment. Based on the results of the PCR, the porin\textsuperscript{365} strain, which had already been noted by Graham to contain an inactive porin gene, did in fact include a faulty porin gene and could be used in the genetic crossings (Graham et al., 2010). The GMR-Gal4 test was used as a control to ensure that all the proper reagents had been added to the PCR mix in case the porin gene did not show up on the gel. Because of its size, there was some concern that if the porin gene was active that it may not show up on the gel because PCR does not work as well on larger gene sequences.

To double-check the results, a new fly prep was prepared with the porin\textsuperscript{365} mutant flies to ensure that the strain did in fact contain a mutant copy of the porin gene. GMR-Gal4 was included on the gel once again as a control. (Figure 5)
Figure 5: PCR of $\text{porin}^{365}$

Lane 1 contains the 10kb ladder, Lane 2 contains $\text{GMR-Gal4}$, and Lane 3 contains a new fly prep and PCR of $\text{porin}^{365}$ mutants.

Figure 5 confirmed the presence a $\text{porin}$ mutant and allowed for the continuation of the genetic crossing.

The initial genotype of the $\text{porin}^{365}$ mutants included a copy of SM6B on the other chromosome. So, with this genotype in mind the $\text{porin}^{365}/\text{SM6B}$ mutant flies were crossed with virgin females with the genotype $\text{GMR-Gal4/CyO}$. Because CyO chromosome has a phenotype of curly wings, the progeny that had this visual balancer were removed so that only $\text{porin}^{365}/\text{GMR-Gal4}$ flies remained. Because neither a $\text{porin}$ mutation nor $\text{GMR-Gal4}$ have a phenotype, removing the unwanted phenotype of flies that were not needed in the continuation of the experiment was essential.
Some of the $\text{porin}^{365}/GMR-\text{Gal4}$ flies were tested via PCR to ensure that they did in fact contain both copies of $\text{porin}^{365}$ and $GMR-\text{Gal4}$ before continuing with the genetic crossing. (Figure 6)

![PCR gel](image)

**Figure 6: PCR Master Mix for $\text{porin}^{365}$ $GMR-\text{Gal4}$ Flies**

The beginning lane is the 10kb ladder, Lane 1 contains the $\text{porin}^{365}/GMR-\text{Gal4}$ prepared with a master mix excluding Taq Polymerase until right before initiating the PCR, and Lane 2 contains the $\text{porin}^{365}/GMR-\text{Gal4}$ prepared with a master mix including Taq Polymerase from the initial mix.

Figure 6 shows that the newly crossed flies do in fact contain both a copy of $\text{porin}^{365}$ and a copy of $GMR-\text{Gal4}$. Figure 6 was also used to test how well PCR would work if there were two different sets of forward and reverse primers, one for $\text{porin}$ and one for $\text{Gal4}$. Based on the success of lane 1, it is clear that both regions of the genome ($\text{porin}^{365}$ and $\text{Gal4}$) could be amplified simultaneously in the same PCR reaction. This concept is important because it is used later to identify potential recombinants that contained both $\text{porin}^{365}$ and $GMR-\text{Gal4}$ on one chromosome. This gel also allowed a
procedural test that could be used to set up a PCR reaction. Lane 1 and 2 both began as a PCR master mix that contained the properly measured quantities needed for each reaction with exception of the Taq Polymerase. Lane 1 was prepared and the Taq Polymerase was added right before it went into the PCR machine and Lane 2 was prepared from a master mix that contained Taq Polymerase already mixed in. As seen on the gel, adding Taq right before the sample begins PCR worked best so that is what is used later in PCR tests that involve simultaneous amplification of both genome regions.

After confirming the $\text{porin}^{365}/\text{GMR-Gal4}$ flies, 54 lines with $\text{porin}^{365}/\text{GMR-Gal4}$ virgin females and $\text{Sco/CyO}$ males were set up. The Sco phenotype is a lack of bristles on the back of the fly and the CyO phenotype, again, is curly wings. These visual balancers were used so that it would be possible identify progeny from the adults. Progeny from these crosses either had no bristles and straight wings or had bristles but had curly wings. It was assumed that some of the progeny’s chromosomes contained a recombined chromosome that possessed both $\text{porin}^{365}$ and $\text{GMR-Gal4}$ on one chromosome.

Because the distance between the two genes was unknown, it was unclear how frequently the recombination should occur or if it would occur at all. Based on the frequency of recombination that was observed, the two genes appeared to be quite a distance apart and therefore recombined quite frequently.

To test whether the flies had successfully recombined, the first 24 vials were selected, and prepared with 2 progeny following the fly preparation steps outlined before. Following this a PCR reaction was run. Because the previous experiment using the master mix without Taq Polymerase was successful, the 24 vials were prepared the same
way for PCR with the Taq Polymerase being added just before initiating the reaction. The results of this PCR are found in Figure 7.

Figure 7: PCR of Potential Recombinants
Lane 1 contains the 10kb ladder and Lanes 2-25 contain potential recombinants from vials 1-24 prepared with the master mix excluding Taq Polymerase until right before initiating PCR

Figure 7 shows that out of the 24 original samples there are 7 potential recombinants that have both \textit{porin}^{365} and \textit{GMR-Gal4} on one chromosome. The lanes that contained both a \textit{porin}^{365} band as well as a \textit{GMR-Gal4} band indicate the potential recombinants. The potential recombinants are noted from lanes 2, 7, 6, 11, 20, and 23 (samples 1, 6, 7, 10, 19, and 22). Based on the number of recombinants, it seems as though the frequency of recombination is somewhat common.

Another PCR was run with new fly preparations of just the 7 potential recombinants to ensure that the lines contained recombinants. Separate PCR reactions
were run with the *porin* primers and the *Gal4* primers to ensure that there was no error or competition. Figure 8 contains the first attempt at a repeat experiment.

Figure 8: PCR of Specific Potential Recombinants
Lane 1 contains the 10kb ladder and the other lanes contain the primer and the sample in parenthesis.

The results of the gel did not come out as expected. The only samples that indicated potential recombinants were samples 7 and 10. Because of this experiment, it was decided to try a new approach. Rather than use pure sample DNA in the PCR procedure, for the next test, the DNA was diluted 1:10 with water. This was done so that a potentially clearer signal on the gel was possible. Figure 9 contains the samples that were diluted prior to the PCR experiment.
The lane set up is identical to that of the previous Figure with the exception that the samples have been diluted prior to PCR.

The dilution partially worked because a better signal occurred compared to that of the previously, but a strong signal from all of the potential recombinants was still not confirmed. The second gel confirmed that sample 10 was in fact a potential recombinant, but it also added sample 19 as a viable selection. There were also extremely weak bands for both the GMR-Gal4 and porin for Sample 1 in lanes 2 and 3, and also Sample 6 in lanes 4 and 5. Samples 1 and 6 were however strong enough to be included in the pool of potential recombinants. Samples 13 and 22 failed to yield bands on both the GMR-Gal4 and porin for either conformation test indicated by Figures 8 and 9 and thus were removed from the pool of potential recombinants. From this point, the remaining 5 samples that were considered potential recombinants were crossed yet again.

Virgin females from samples 1, 6, 7, 10, and 19 were crossed with males from the UAS-Bax/TM6Sb line and allowed to grow. The goal for this cross was to produce progeny flies that contained porin\textsuperscript{365} and GMR-Gal4 on one of the homologous chromosomes and UAS-Bax on the other.
After the conclusion of the cross, “rough” eye flies were not found among the progeny of the potential recombinants and the \textit{UAS-Bax} flies. As a confirmation that the cross was successful, other progeny flies were examined and noted to contain the correct phenotypes from the cross. The flies that were supposed to contain “rough” eyes should not have curly wings or stubble in their phenotype, but all of the flies (greater than 200 flies) that lacked these phenotypic markers did not have “rough” eye.
Discussion

Conformation of Inactive porin Strain

The supposed inactive porin strain, porin\textsuperscript{78}, that was initially used was shown by PCR to produce a signal equivalent to that of the wild type porin\textsuperscript{27} strain. The wild type porin allele is based on the expected size of the PCR product. An intact gene yields around 3 kb PCR product while an inactive form is smaller. The inactive porin is smaller because a segment has been deleted and therefore is not able to fully transcribe a porin mRNA. Based on Figure 4, it is possible to note that rather than containing a smaller porin gene, porin\textsuperscript{78} actually contained the same size gene as that of porin\textsuperscript{27}. Based on the PCR results, it was concluded that the porin\textsuperscript{78} line, which contain a supposed knockout version of the porin gene, had an intact porin allele.

From Figure 4 as well, it is shown that the porin\textsuperscript{365} line does in fact contain a smaller gene than 3 kb. The porin\textsuperscript{365} gene is around 1.5 kb, which shows that this line does contain an in-active form of porin. For this reason, porin\textsuperscript{365} was chosen as the initial porin strain over porin\textsuperscript{78}.

PCR Using Multiple Primers

Using multiple sets of PCR Primers was not initially discussed in the original protocol, but this was a necessary test in order to confirm the presence of GMR-Gal4 and
porin\textsuperscript{365} on one chromosome from many samples. The potential issue with this test is whether or not the original primers would interfere with one another and therefore create a dimer rather than the PCR product of choice. The PCR from Figure 6 showed that the individual primers that were being used to amplify GMR-Gal4 and porin did not in have an affect on one another and therefore could be used in a multi-gene PCR test.

**Formation of a GMR-Gal4 and porin\textsuperscript{365} Recombinant Line**

Initially, there was a bit of uncertainty as to whether a GMR-Gal4 and porin\textsuperscript{365} line was even possible because the gene separation distance on the chromosome was unknown. With that being said, a set of genetic crosses was still devised to potentially create this recombinant. After completion of the series of genetic crosses, PCR testing was able to confirm the presence of both GMR-Gal4 and porin\textsuperscript{365} in flies that also contained a phenotypic balancer. The use of this balancer was to ensure that both GMR-Gal4 and porin\textsuperscript{365} were present in the same fly and on the same chromosome. Out of the 24 samples that were tested, PCR confirmed that there were originally 7 potential recombinants. Statistically, this percentage was much better than what was originally expected. Out of those 7 that were selected and re-tested, only 5 were able to consistently produce both PCR products. The other 2 samples that were originally believed to be potential recombinants were discarded because on two separate fly preps they only produced a porin\textsuperscript{365} band. It is possible that in the original fly prep, since 2 flies are used, that one contained a chromosome with porin\textsuperscript{365} and a visual balancer on its other chromosome while the other fly contained GMR-Gal4 and a visual balancer. In this case,
the signal from the PCR would contain both GMR-Gal4 and porin\textsuperscript{365} but would not contain a recombinant chromosome.

It was also important to note that the frequency of the formation of potential recombinants appeared to be quite high because that would increase the chances that, when the next cross between the potential recombinants and the UAS-Bax/TM6RSb flies occurred, the parents were in fact recombinants. If there were a low frequency of recombination then it would be more difficult to find progeny flies that contained both a recombined chromosome with porin\textsuperscript{365} and GMR-Gal4 as well as a UAS-Bax chromosome.

Porin Bax Relation

The porin\textsuperscript{365} and GMR-Gal4 UAS-Bax recombinant flies failed to yield the phenotypically expected rough eye as noted in the GMR-Gal4 UAS-Bax cross. The overall health of the eye was normal in the porin\textsuperscript{365} mutants even when localized Bax expression occurred. This seems to contradict the hypothesis that VDACs negatively affect the formation of the Bax channels and therefore negatively affects the process of apoptosis. In fact, the results seem to point to a different idea that an active porin channel may actually enhance apoptosis since rough eyes are observed in GMR-Gal4;UAS-Bax flies that have wild type porin alleles.

If this is the case, then future studies should be geared toward creating a Drosophila melanogaster line that includes two inactive copies of porin. If porin were critical for apoptosis to occur, then the eye would remain ordered even in the presence of
an active Bax protein. This potentially opposite relation than what was originally outlined could indicate a dependence that Bax has on porin.

On the contrary, it is possible that the original relation still exists, but that the UAS Gal4 system is not working properly. Because of the number of crosses and generations of flies that had to be cultivated that contained the GMR-Gal4 gene, it is possible that, through random recombination, this gene has become less potent and therefore less active in the UAS Gal4 system. With a less active version of Gal4, there would be a decrease in Bax activation and therefore a decrease in the fly eye cell death.

Future studies to prove the Gal4 gene’s potency could include doing reverse transcriptase PCR. This process allows for the amount of mRNA of a certain tested gene, in this case Gal4, to be quantified. If the amount is minimal then the potency of the Gal4 has been reduced in the line and this could be the reason that the Bax activation did not yield a “rough” eye. If the quantity of Gal4 is at the correct level and the potency has not decreased yet the eye is more ordered than that of the GMR-Gal4; UAS-Bax cross, then the relation of porin and Bax is possibly the opposite than that of the original hypothesis. Another possible future test is to cross the line with another line that contains a UAS gene that does not relate to Bax or apoptosis and where the activation of that gene is phenotypically visible. If the gene is activated and the phenotype is observed, then it is possible to note that the GMR gene has maintained its potency and remains active. If the new UAS gene has not been activated then it is also possible to note that the potency has potentially been reduced and therefore is the reason for the lack of Bax activation in the porin mutant eye.
LIST OF REFERENCES


