CLONING HEMAGGLUTININ-TAGS IN MITOCHONDRIAL DNA

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INTRODUCTION
Mitochondria are a crucial organelle in the cell. They not only function as the powerhouse of the cell by creating most of the cell’s ATP, but they also have a large role in signaling, cell death, and cellular differentiation. They also have a separate genome from the rest of the cell that is inherited maternally. When mutations in this mitochondrial DNA (mtDNA) occur, there are several devastating effects including Alzheimer’s disease, ALS, Parkinson’s disease, and Huntington’s disease [1]. The Palladino lab focuses on a mutation that directly impacts subunit 6 of the ATP Synthase protein in the mitochondria, commonly referred to as ATP6. This mutation is very closely linked with Maternally Inherited Leigh’s Syndrome (MILS), Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP), and Familial Bilateral Striatal Necrosis (FBSN) [2]. Currently, mitochondrial diseases affect 10-15 people in every 100,000 and majority of the treatments are ineffective.

The Palladino lab has adapted a version of an ATP6 mutation that is implemented in a fruit fly (Drosophila melanogaster) in order to test treatments for these diseases in vivo. The fruit fly is used because it has a very well understood genome and it is easy to control its mating. The current technique for treatment of the fruit fly involves injection of new DNA into the mitochondria, which is no easy task. In order to get the DNA into the mitochondria, a method called allotropic expression is used. Allotropic expression is when DNA is transcribed into RNA in the nucleus and then transported into the mitochondria where it is translated into protein [2]. In order to see if the correct protein is being expressed in the mitochondria, there needs to be an epitope tag attached to the imported DNA that allows the resultant protein to be distinguished from the naturally occurring protein. The DNA that is being imported translates into ATP6 with an epitope attached to the end of it.

The current plasmid DNA that has been tested included a myc-tag at the end of it. When tested for presence in flies, it was not visible. This led to some concern, as it was unclear if the import method was functioning or not. After much research, Dr. Palladino decided that using a Human Influenza Hemagglutinin (HA) tag would be the best approach as it has been shown to work consistently. HA is a nine amino acid sequence that was derived from the Human Influenza virus [3]. The plan, simply, was to remove the myc-tag and put the HA-tag in the same exact place.

OBJECTIVE
There are two main objectives of this project. The first is to design a HA-tag based on previously used versions and have it successfully inserted into the Palladino Lab’s previously existing plasmid. The second objective is to test the presence of the HA-tag in the mitochondria of the in vivo system by using a Western Blot.

HYPOTHESIS/SUCCESS CRITERIA
If the first objective is successful, the sequence of the HA-tag should match what was originally designed. The second objective would be deemed successful if the HA-tag is clearly visible on a Western Blot after several generations of mating.

METHOD
The initial step in the process was to find variants of the HA-tag that had been used in prior research. This involved searching through PubMed articles and looking through different databases that dealt with DNA. Once the sequences were found, they were adapted using DNASTAR Lasergene in order to function in the Drosophila melanogaster mitochondrial genome. Once adapted properly and repeatedly checked for errors, primers were designed and ordered for Polymerase Chain Reaction (PCR). PCR is a process that replicates specific DNA sequences exponentially by using a bacterial DNA polymerase that functions at higher temperatures than human DNA polymerase (Promega Corporation, Madison, WI). After the PCR cycle, the product was loaded into a 1% agarose gel for gel electrophoresis, which separated the PCR product into bands based on size.

Once the band size is confirmed to be correct, the HA-tag along with the lab’s old plasmid was digested with the restriction enzymes Xba1 and Kpn1. This allowed the HA-tag to fit right into where the old epitope tag had been. After the HA-tags had been inserted, they were attached to the plasmid using a DNA Ligase in a process called Ligation. The next step was to give the DNA to E. coli in order to amplify the quantity of DNA, which will be needed in the next step, the miniprep. The miniprep is a process that takes the resultant from the transformation and purifies it to plasmid DNA in small quantities, which allow for diagnostic tests. The first diagnostic test was another digestion. After the digestion was run, the digestion mix was loaded into a 1.2% agarose gel for gel electrophoresis. The band size was confirmed with the model, which led to the more significant diagnostic test: sequencing (GENEWIZ, South Plainfield, NJ). The sequence of the HA-tag was confirmed and the miniprep samples were ready for a similar process called midiprep. Midiprep is a very similar technique to miniprep except the overall size is larger, creating DNA concentrations high enough for injection into flies (Genetic Services, Inc, Sudbury, MA).
Upon receiving the flies after injection, they were immediately implemented into a mating scheme. This mating scheme consists of 8 generations that stabilize the fly stock in addition to adding the mitochondrial disease and the prospective treatment for it. These matings are still ongoing. For each generation, there are two vials of flies used, each with anywhere from 6-20 flies depending on how prolific the previous generation is. After the stock is stabilized, females will be isolated and their thoraces will be taken and be prepared for use in a 40% acrylamide gel. The gel separates proteins in the same manner that agarose gel separates DNA. Once the gel has been run, the protein on it is transferred to transfer paper that is then treated with antibodies that are specific to the ATP6 protein. The transfer paper is then exposed with x-ray film, resulting in the Western Blot. The Western Blot is analyzed to see if the HA-tag is present.

RESULTS

Results for this experiment were analyzed throughout the entire process, with success required in each step before the next step could be started. The first crucial result was with PCR. Confirming the correct band size was crucial because it showed that the HA-tag was designed properly. The result from PCR is shown in Figure 1 below.

![Figure 1](image.png)

Figure 1. PCR result from HA-tag, showing band size was around the expected length of 117 base pairs. The column on the left shows the scaling of the gel with units of base pairs.

As the fly matings are still going on, there are no results for the Western Blot yet. Bands are expected to appear around 26 kilodaltons (kDa) and 30 kDa, resulting from the naturally occurring ATP6 protein and the addition of the HA-tag respectively as shown in Figure 2.

![Figure 2](image.png)

Figure 2. Predicted Western Blot results with scale on left in kilodaltons (kDa). The bottom band is from the naturally occurring ATP6 protein while the top band is the ATP6 protein plus the HA-tag.

The true results are still unknown at this point in time but should be attained in the coming months.

DISCUSSION

The results of this project are not complete yet, but the first objective has been successfully completed. The molecular cloning was checked several times throughout the entire process in order to confirm that everything was going as anticipated. The PCR worked flawlessly as did all of the next steps.

Objective two has not been completed yet, but there are signs that it is going well. There are only two generations remaining in the mating scheme and all of the prior generations have been healthy and have been showing the same phenotypes that the mating scheme says they should.

There are a few factors that have limited this study so far. The first being the amount of time needed for the mating scheme to be completed. It has taken the entirety of a semester and there are still two generations to go. With two weeks per generation and then another week to collect flies for the next generation, the time truly adds up. The HA-tag is also only being expressed in fruit flies and not in humans yet. There are still many steps that need to be taken before application in humans can even be considered.

Should the HA-tag be seen on the Western Blot, the Palladino lab would have confirmation that everything that has been done previously working with mitochondrial import would be correct. The confirmation would allow for further development and application into human cells and later, humans in vivo, thus creating an effective treatment for mitochondrial diseases.

ACKNOWLEDGMENTS

I would like to thank my mentor Atif Towheed as well as my PI Michael Palladino for giving me the opportunity to do research in this wonderful lab. I would also like to thank Aaron Talsma for teaching me all that I needed to know about fruit fly phenotypes and mating. To everyone else in the lab that helped me along the way, thank you.

REFERENCES


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