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Earthworm Remyelination Gene Expression Analysis

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ABSTRACT

An earthworm has the unique ability to regenerate its body once it has been cut or damaged. As part of this regeneration they may possess the capability to repair their myelin sheath. This is a very desirable characteristic considering most organisms can not. The myelin sheath surrounds the ventral nerve cord and is essential in the signaling of the nervous system. What is unknown about the earthworm is which genes are present in its myelin and whether they are involved in this suspected remyelination process. Through the dissection of earthworms, RNA purification, cDNA synthesis, and RT-PCR, the genes involved and their corresponding roles in the regeneration process can be found. The RT-PCR process quantifies the amount of a targeted DNA sequence, and through this quantification it can be discovered which genes play a possible role in remyelination. Once the involved genes are discovered it will allow for better understanding of the regeneration process of the earthworm. Through the course of this experimentation, the genes: Glutamine Synthetase, 14-3-3, Translationally Controlled Tumor Protein (TCTP), and Actin Related Protein can be confirmed to be found in the earthworm genome and presumably up-regulated during regeneration. More trials are necessary to confirm the latter and further research into this may lead to medical advances in repairing diseased and damaged nervous systems of humans.

INTRODUCTION

A gene is a molecular unit of heredity in a living organism. These genes make up a cells DNA, which then express an individual's traits and characteristics. It may not be obvious, but earthworms and mammals share a large majority of common DNA. Thanks to evolution, when studying these earthworms, a lot can be concluded about humans as well. One characteristic is especially interesting when it comes to its convergent evolution in mammals and earthworms. This is the myelin sheath. Myelin is an insulating layer around the axon of an organism. This is important because of its essential role for the proper functioning of a nervous system. The myelin layer does not stretch along the entire length of the axon; there are gaps in the myelin layer called nodes of Ranvier (Jahn, 2009). These nodes are what make the electrical current down the axon increase. They allow for an intense influx of sodium ions, which propagate the electrical current down the axon (Long-Term Maintenance, 2014). This signaling process is vital in almost every organism, but past that is where the differences begin to appear.

The myelin sheath is wrapped around the axon by two different types of cells, this depending on what part of the nervous system being looked at. In humans or other mammals, in the central nervous system, the myelination of an axon is done by oligodendrocytes, and in the peripheral nervous system it is done by Schwann cells (Aggarwal, 2013). In earthworms, on the other hand, it is thought to be a Schwann like cell that does the myelination. This leads to the belief that the proteins involved may potentially be different in earthworms and other mammals as well. Myelin, as known, is mainly composed of lipids, with some embedded proteins. About 70%-75% of the dry weight of the myelin sheath is lipids (Jahn, 2009). This is comparable to a human cell membrane which only has a 25%-30% lipid composition. This all becomes even more significant when looking at the demyelination process. The degradation of myelin is a

debilitating part of many diseases, and in most organisms once it is harmed it cannot be successfully repaired (Taveggia, 2010).

Earthworms are such an interesting subject to study because they possess the unique quality known as regeneration. They can regenerate the cells making up their axons once they have been cut or destroyed, thus allowing either the healing of a wound or, in certain cases, the reproduction of a new tail entirely (as long as the cut is below the clitellum). The underlying thought behind all of this is whether or not earthworms can reproduce their myelin sheath along with their axon, and if so, what makes their myelin different than that of a mammal, which does not have this ability. By studying the features of earthworm myelin there lays the potential to answer some of these questions.

It is because of these fascinating questions that arises the unfamiliar aspects of earthworms which are being studied here. There is currently not a lot known about the nervous system of the earthworm to begin with. Looking closer at its regenerative feature, it remains unknown which genes make up its own specific myelin sheath and further which ones exactly are involved in the regenerative process. There has been research into the myelin proteins of mammals for the purpose of remyelination but not yet on earthworms. It is the goal of this experiment to determine these genes and verify their role in suspected remylination. Once the genes present here have been identified and it is established how they change during regeneration, they can be compared to those studies done on other species to help determine their differences.

This specific project is especially important because it not only allows us to investigate the differences between humans, or other mammals, and the earthworm but how these differences can help advance medical prospects. Mainly, this research aims explore genes

differences that enable these worms to regenerate certain features. If indeed earthworms are able to regenerate their myelin as well as their axons, it can be explored what specific genes are responsible for this aspect. Once this is known, it can be applied to human research to determine protein differences in different species' myelin. Even further this research could someday lead to help in human myelin regeneration by reversing the effects of demyelinating diseases such as multiple sclerosis or leukodystrophy.

MATERIALS AND METHODS

Isolation of RNA:

The procedure began with the dissection of an earthworm. Each worm was dissected using a dissecting scope, pins and razor blades/scalpels. The worm was cut open on the dorsal side as to not harm the axon or myelin sheath. The organs were then moved aside and the axon was removed using a tweezers. A picture of the axon during dissection can be seen in Figure 1. The axon was then isolated so that its RNA could be extracted and purified. 50mg of the obtained axon tissue was homogenized and added to 500 µl of VRX buffer. The homogenized cells were centrifuged for 2 minutes. The liquid was then transferred to an RNase free tube, where it was purified. One volume of 98% isopropanol was added to the one volume of the homogenized sample and buffer and vortexed. The mixture was placed into an RNA mini column and centrifuged for one minute with the liquid in the collection tube being discarded. The column was centrifuged again and the flow through was discarded again. The sample was then washed twice with 500 µL of WS buffer by centrifuging for one minute, the flow through being discarded both times. To get rid of the residual buffer the sample was centrifuged for three more minutes and the final flow through was discarded once again. The column was transferred

to an RNase-free tube and the RNA was eluted from the column by adding 50 µL of RNase-free water to the column and centrifuging for one minute. The purified RNA solution in the flow through was then stored at -70°C.

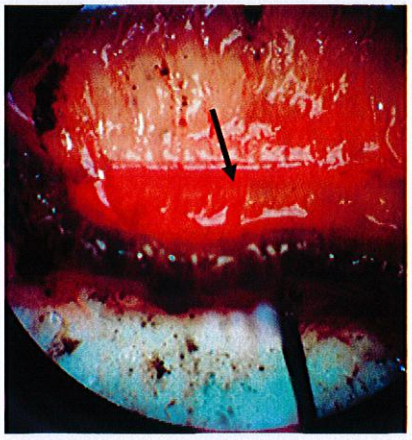

Figure 1- Dissecting Scope Magnification of Inside an Earthworm
The arrow indicates the axon which was extracted.

Gel Electrophoresis and Spectroscopy:

In order to confirm the presence of RNA in the obtained sample, an Agarose gel electrophoresis was preformed. A one percent gel was created with 0.3g of Agarose and 30mL of TAE solution. 5µL of the RNA sample along with 1µL of 10x loading dye and 4µL of water were added to the well in column two. A 10µL control sample of ladder was run in column one. The results are shown in Figure 2. RNA was confirmed present in the sample when comparing column 2 to control column 1.

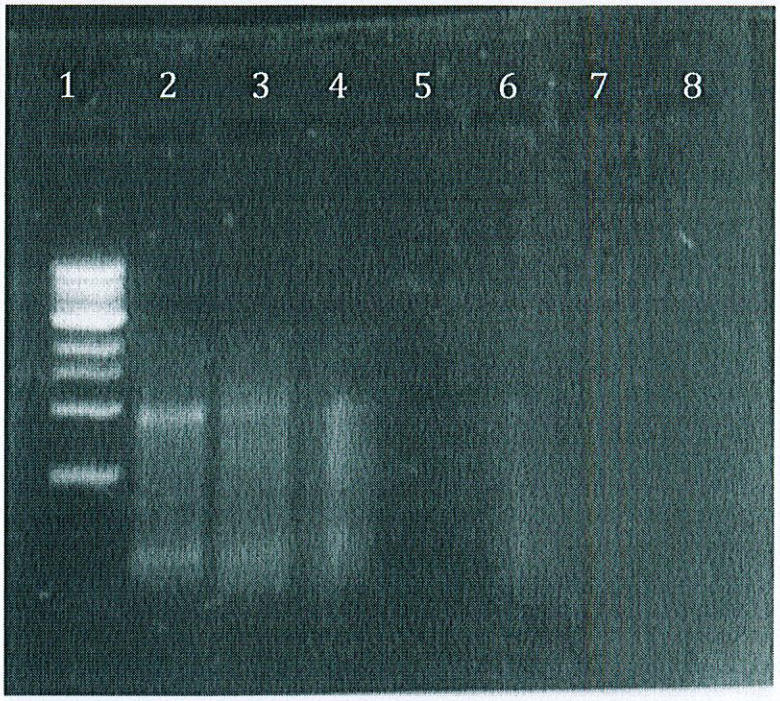

Figure 2- Gel Electrophoresis Results
Presence of RNA is confirmed by our sample in column 2 as compared to column 1. (All other columns irrelevant)

Once the presence of RNA was confirmed, cDNA was synthesized from the RNA. 10 μg of the RNA was placed into a PCR tube with 0.5 μL of oligo dT. DEPC water was added to the tube to make the finale volume 12.5 μL. The tube was put into the thermal cycler for five minutes at 70°C. After the sample had gone through the thermal cycler, it was placed on ice for five minutes. 5 μL of M-MLV 5x buffer, 5 μL dNTP mix (with each nucleotide at 2.5 mM), 1 μL RNasin, and 0.5 μL M-MLV RT were added to the solution and mixed by pipetting up and down. The tube was then incubated for 60 minutes at 40°C and then stored at -70°C.

After synthesizing the cDNA, its concentration had to be evaluated. This was done using spectrophotometry. The obtained cDNA sample was evaluated using the wavelengths 260 nm and 280 nm which are employed to detect DNA in a solution. This provided the actual concentration of the cDNA sample which was 1.311μg/μL.

Primer Development:

Once the cDNA was synthesized and evaluated, the preferred genes had to be chosen. The proteins that were selected for analysis in RT-PCR were selected from a list that was created by Olaf Jahn, in his paper *Myelin Proteomics: Molecular Anatomy of an Insulating Sheath.* This list was comprehensive of all the proteins involved in the initial myelination process. From this paper, some of the most prominent genes for prospect in remyelination were selected; Glutamine Synthetase (GS), 14-3-3, Actin Related Protein (ARP), Translationally Controlled Tumor Protein (TCTP), and 14-3-3 Tau. Lumbrokinase was also chosen to observe as a positive control as it should be found in all earthworm cells. The negative control was no gene. From here, primers needed to be developed in order to test for the presence of said genes. The gene sequences for each of these genes were found on the National Center for Biotechnology Information (NCBI) website. Once the sequences were obtained they were "BLASTed" against the *Lumbricus rubellus* (earthworm) genome. This allowed for the procurement of their location and corresponding sequence in the earthworm. The sections of each gene with the most correlation in the earthworm genome were then analyzed. The nucleotide sequence was examined, sent, and developed using a program called OligoAnalyzer on the Integrated DNA Technologies (idtdna.com) website. This is where the final forward and reverse primers were created. The primers that were used in RT-PCR required a minimum of 13 nucleotide base pairs and had to have a melting temperature of 55-58°C. The final forward and reverse primers were:

Lumbrokinase R: 5'-TCCATCCATGCAGTGAGCA-3'
Lumbrokinase F: 5'-TATCCGTCCGCAGGAAGT-3'
Glutamine Synthetase R: 5'-GCTTGGGCCAGCCAAA-3'
Glutamine Synthetase F: 5'-TCCATGGTTTGGAATTGAACAGGA-3'
14-3-3 R: 5'-TATCCCTAAGCAGCTGCATGATTA-3'
14-3-3 F: 5'-AACTTCTCCGTGTTTTACTACGAGATT-3'
Actin Related Protein R: 5'-GCTTGGACTTCCGCGTCT-3'
Actin Related Protein F: 5'-TCACAGATTCGTAGAATAATTGCACAC-3'

Translationally Controlled Tumor Pro R: 5'-CGACTTGATGTGGGTCATGTAGT-3'
Translationally Controlled Tumor Pro F: 5'-AGTTGCGTACAGACACTTTCG-3'
14-3-3 Tau R: 5'-CCAAACTGTGTCCGGTCAGA-3'
14-3-3 Tau F: 5'-TCGGATGCGGGAGAGG-3'

Real Time Polymerase Chain Reactions (RT-PCR):

The cDNA that was originally synthesized was used in Real Time Polymerase Chain Reaction (RT-PCR) analysis for the above proteins. This was done on an Applied Biosystems One Step RT-PCR Machine by following the KAPA SYBR FAST RT-PCR protocol (KAPA, 2013). The only change being the numerical values. The amount of solution in each reaction was cut in half in order to conserve cDNA and KAPA 2x Master Mix solution to allow for more trials. 0.4μL per reaction of each corresponding 10μM forward and reverse primer was mixed together per reaction to reduce pipetting error. This was then mixed with 9.2μL of a master mix that was made from 4μL of water, 5μ of KAPA SYBER FAST 2x Master Mix, and 1μL of cDNA, each per reaction. The total of 10μL was then placed in its own well on a RT-PCR tray. This was done for every gene including the positive control, Lumbrokinase. The negative control was simply 10μL of Master Mix and no primers. The RT-PCR cycled between 95°C and 60°C in order to denature, anneal, and extend the DNA. This process took about 90 minutes to complete all cycles. From there a graph was produced verifying the presence of the genes in the sample.

After the RT-PCR confirmed the presence of the desired genes in the earthworm DNA, we began the comparison to a regenerated worm's axon. Twelve worms were cut on their dorsal side just enough to cut their axon but not enough to slice the worm in half. The cut was made just below the clitellum of the worm so that the worm would be able to regenerate. Four of the twelve worms survived the procedure and were then dissected at different points during their regeneration progress. Worm one was dissected three days after being cut, worm two was

dissected ten days after being cut, worm three dissected fourteen days after being cut, and worm four dissected seventeen days after being cut. The axons of each worm were then isolated and extracted from both sides of the cut where the worm was expected to be remyelinating. These sites can be seen in Figure 3. Another sample of each axon was taken from farther up towards the head of the worm, as a control sample. The control sample should not have been affected by the cut. These samples were then taken through the same RNA purification, cDNA synthesis, and RT-PCR processes as the original sample. The amplifications produced from the genes in the regenerated axon segments and the control axon segments were then able to be compared from their normalization to corresponding Lumbrokinase samples.

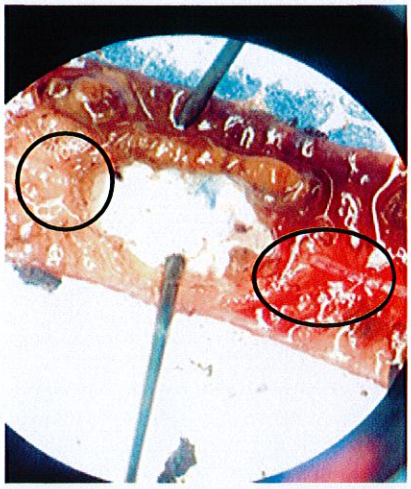

Figure 3- Dissected Earthworm 3 Days after Being Cut
Image was taken through a dissecting scope. The cut is visible. The circles indicate where myelin samples were taken from.

RESULTS

From the RT-PCR, amplification plots were produced showing the amplification and presence of the genes in the obtained DNA samples. These plots provided amplification values from the cycle number the genes were registered during. The first graph in Figure 4 presents the results of the original run showing that all genes are certain and accounted for in the DNA of an uninjured earthworm. In the two initial trial runs the amplification numbers were irrelevant as the intent was only to confirm that the genes were in fact located in the cDNA. The heights of the amplification lines on the graph are irrelevant as well and are of no significance to the results of this experiment. The genes presences were confirmed by the initial rise of each line in the original run and in a second run showing very similar results.

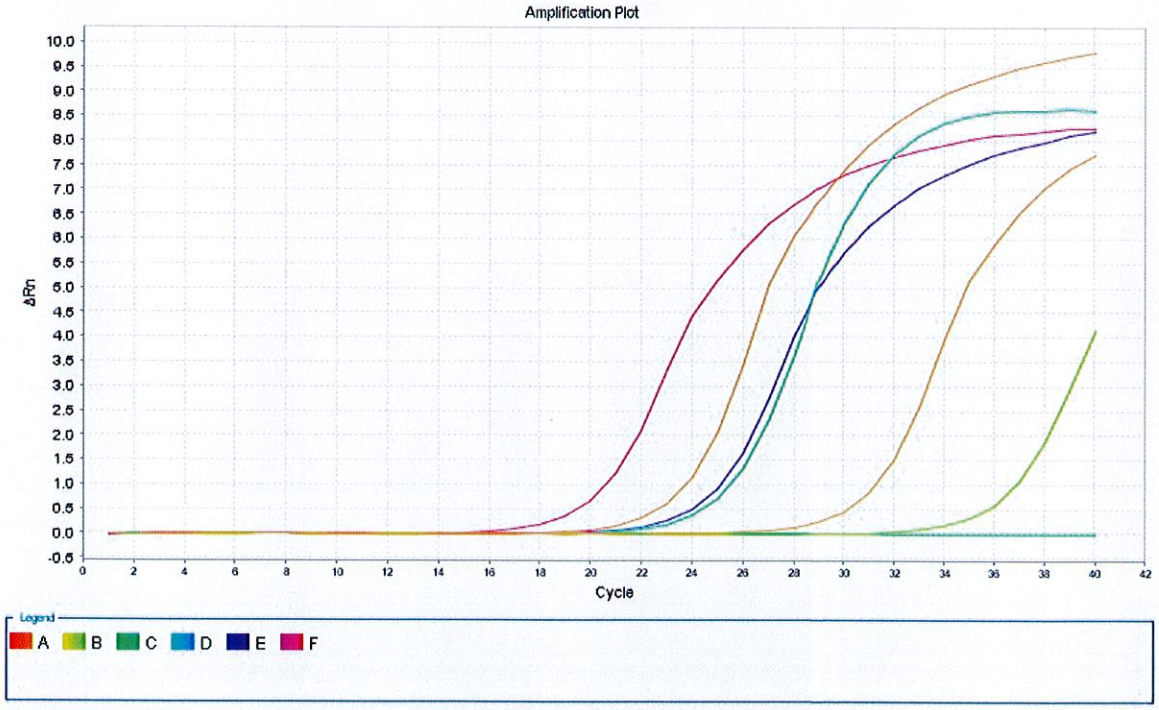

Figure 4- Initial RT-PCR Amplification Plot of Proteins in Un-cut Worm
In order of highest to lowest amplification (from left to right)
TCTP, 14-3-3, Lumbrokinase, Glutamine Synthetase,
Actin Related Protein, 14-3-3 Tau, Negative Control (No Amplification)

From there, the results of the RT-PCR amplification plots for the regenerated segments of the earthworm axon were analyzed. The amplification numbers (aka cycle numbers) of the genes in the regenerated segments were compared back to the amplification numbers of the same genes in the control segment of the same worm. This was done using Lumbrokinase as a normalization point. The reason the normalization was necessary was to account for any differences in the concentration of the two seperately synthesized cDNA samples. In order to determine whether there was any up-regulation or down-regulation of a certain gene, the cycle number of amplification of an individual gene was compared back to the cycle number of Lumbrokinase from the same cDNA sample. These cycle numbers for comparison are listed in Table 1. Those numbers are then compared mathematically by taking the difference between a genes amplification number and the corresponding amplification number of Lumbrokinase in the sample cDNA sample. A visually simplified version of this can be seen in Figure 5. The up-regulation can be seen when comparing the distance between the amplification of a gene from the cut/ regenerated axon and its corresponding Lumbrokinase and the distance between the amplification of a gene in the control site and its corresponding Lumbrokinase. A greater distance from the cut/ regenerated sample than in the control sample shows up-regulation.

	Cycle Number					
Gene	Worm 1 Cut Site	Worm 1 Control Site	Worm 2 Cut Site	Worm 2 Control Site	Worm 3 Cut Site	Worm 3 Control Site
Glutamine Synthetase	15.3	36.95	33.22	31.45	35.49	34.23
14-3-3	26.23	31.9	28.23	29.49	33.82	31.52
TCTP	23.75	26.13	24.9	26.11	26.81	27.04
14-3-3 Tau	36.98	33.31	35.91	34.54	35.65	33.89
ARP	27.65	31.02	32.66	31.99	37.09	-
Lumbrokinase	32.56	31.92	32.76	31.59	32.002	31.18

Table 1- RT-PCR Cycle Numbers Where Threshold Was Met for Regenerated Worms and Controls
This table shows the raw data that was collected after the cut and uncut worm samples were ran through the RT-PCR. The cycle number corresponds with the amount of cycles that the gene had to go through in order for the machine to register the gene product.

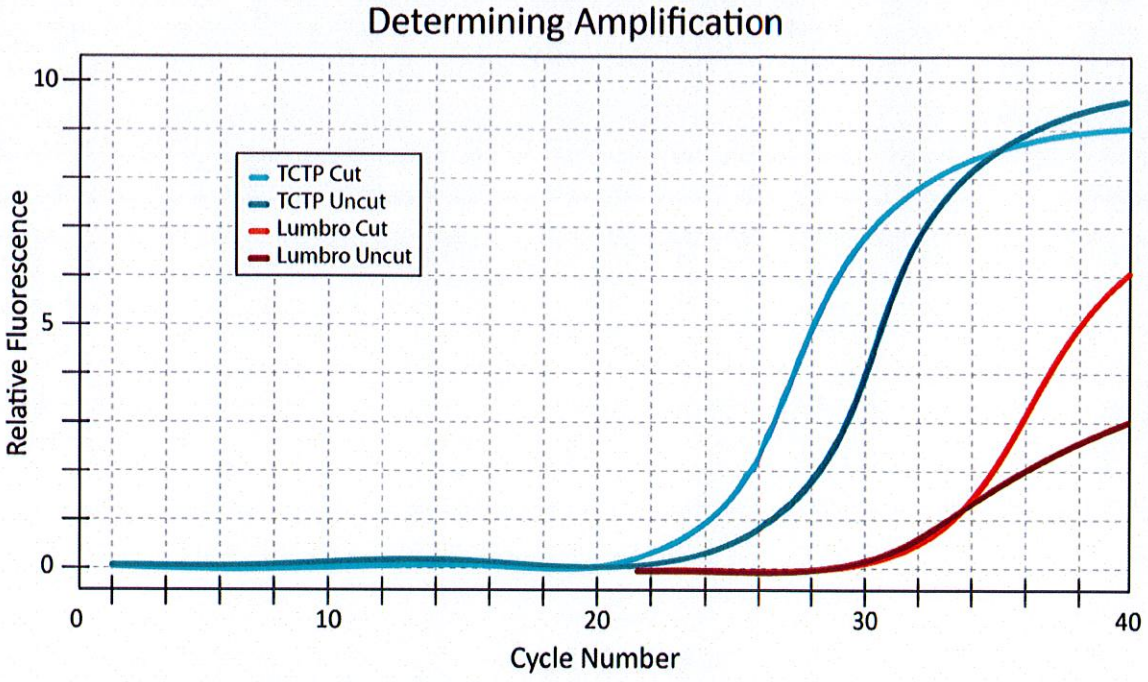

Figure 5- Determining the Amplification of Selected Genes
This graph depicts the up regulation of Translationally Controlled Tumor Protein as compared to the control, Lumbrokinase. The cut cDNA of the earthworm has a greater amplification distance from Lumbrokinase then the control cDNA.

The given cycle numbers in Table 1 were then used to calculate final up-regulation and down-regulation numbers. These numbers are important in determining the amount of up-regulation or down-regulation of a gene so that it may be evaluated and compared to other genes. These numbers were calculated using the "delta delta Ct" method of analysis (6 Quantitative PCR, 2014). The equation behind this was: Regulation Direction = $2^{(\Delta cut - \Delta control)}$; "Δ" being the difference between the intended gene amplification number and its corresponding Lumbrokinase sample amplification number. The final numbers from these calculations can be seen in Table 2.

Gene	Relative Change	Relative Change	Relative Change
	3 Days	10 Days	14 Days
Glutamine Synthetase	+211000	+1.5	+1.4
14-3-3	+32.7	+1.1	+2.8
TCTP	+3.3	+1.0	+0.7
14-3-3 tau	-8.2	-1.1	-1.9
Lumbrokinase	1.0	1.0	1.0

Table 2-Calculated Up-Regulation and Down-Regulation of Genes
Regulation determined by normalization to Lumbrokinase and "delta delta Ct" analysis. Positive numbers indicate up-regulation. Negative numbers indicate down-regulation. Lumbrokinase is the control point.

DISCUSSION

The results of this experiment show that the genes; Glutamine Synthetase, 14-3-3, and Translationally Controlled Tumor Protein (TCTP) were all up-regulated during the regeneration of earthworms when normalized to their corresponding Lumbrokinase samples. 14-3-3 Tau was shown to be down-regulated. Actin Related Protein was not considered for the final up-regulation/ down-regulation results because of incomplete data. The regulation numbers in Table 2 also show that these up-regulated genes were most up-regulated right after the cut was made. As the regeneration process proceeded, the amount of up-regulation of the genes decreased from the initial number. Glutamine Synthetase showed an extremely large level of up-regulation three days after the cut was made. Since this is such an extreme outlier, its information can not be reliable without further trials to confirm it. This is also true for all of the genes analyzed in this experiment.

In order to confirm that the final results of this experiment are truly significant, more trials are essential. Only one, maximum two, RT-PCR analysis trials were done for each of the axon/myelin samples due to limiting resources and limited time. With access to excess KAPA SYBER FAST 2x Master Mix, excess primers, and excess earthworms (in order to synthesize

more cDNA), it would be possible to run 3, 4, or even more trials to confirm that all trials are in correlation. This would also allow for averaging between trials to acquire more accurate numerical values for the up-regulation or down-regulation of the genes during regeneration. Aside from being necessary to confirm the results of this experiment, more trials are also necessary to eliminate any sources of error. The Applied Biosystems One Step RT-PCR Machine used during this process had not been utilized in a few years and thus had not been re-calibrated since. Re-calibration of the machine would have allowed for even more precise readings of the data. Another possible error source was in the pipetting. When using such small amounts of liquid, the accuracy of the pipet is brought down. Inaccuracy in measuring such small volumes is probable when looking at the varying heights in the amplification plots. If pipetting error occurred during the addition of cDNA or primers, the machine would have a harder time recognizing the genes presence and throw off the obtained amplification numbers. More trials would eliminate this possibility when the amplification numbers from all trials were found in correspondence.

If this research were to be continued in the future with access to more materials, dissections of the regenerating worms would be done each day of the regenerating process rather than once per week. This would allow for a clearer picture of the process in action. RT-PCR analysis of each day of the regeneration would also show how the up-regulation of the genes changes over time. Another option would be to explore additional genes that play a role in myelination to determine whether or not they have similar results. It would be interesting to investigate deeper into which myelinating genes are involved in the regeneration process. All of these concepts are contributing factors to this area of research and are important when considering not only these results, but further study in the future.

CONCLUSION

In summary, the procedure and experimentation was found to be successful considering the final results of the RT-PCR analysis. Primarily, it can be confirmed that the proteins; 14-3-3, Translationally Controlled Tumor Protein (TCTP), Lumbrokinase, Glutamine Synthetase (GS), 14-3-3 Tau, and Actin Related Protein (ARP) are all present within the myelin sheath surrounding the axon in earthworms. According to the obtained data, all of the genes involved in myelination that were analyzed here were up-regulated at the beginning of the regeneration period with the exception of Actin Related Protein and 14-3-3 Tau. Because of this up-regulation, one can be led to believe the genes: TCTP, 14-3-3, and Glutamine Synthetase's, roles are important when repairing the axon and supposedly the myelin sheath. More information is necessary in order to confirm and enhance these results before they can be recognized as significant.

ACKNOWLEDGMENTS

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