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Post-Transcription Regulatory Pathway in *C. elegans* using Library Mos-1 mediated single copy insertion

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**Post-Transcription Regulatory Pathway in *C. elegans* using Library *Mos-1*
mediated single copy insertion.**

A Major Qualifying Project

Submitted to the Faculty

of

WORCESTER POLYTECHNIC INSTITUTE

In Partial Fulfillment of the Requirements for the

Degree of Bachelor of Science

By:

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Abstract

Early embryogenesis and cell fate specification of *Caenorhabditis elegans* is mainly driven by post-transcriptional regulation, involving RNA binding-proteins that bind to the 3' UTRs of maternal mRNAs. Regulation of this binding activity is crucial to the maturation of the zygote since this process ensures proper formation of the body plan. The goal of this project is to understand the regulation of different 3' UTRs by different RNA binding proteins. To study this regulation, it is necessary to study the expression patterns driven by 3'UTRs using transgenic strains. To achieve this, we used a library MosSCI approach to generate multiple reporter strains in a faster way.

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I would like to thank Professor Sean Ryder for allowing me to work in his lab and providing me with the information and the tools to aid in the completion of this project. I would also like to thank Ebru Kaymak for teaching me all of the techniques required to carry out all of the experiments performed, as well as guidance throughout all lab work. Finally, I would like to thank Professor Destin Heilman for providing assistance and feedback throughout the duration of my project.

Introduction

This MQP coincides with the work performed by Dr. Sean Ryder and doctorate candidate Ebru Kaymak at the University of Massachusetts Medical School on the nematode worm *Caenorhabditis elegans*. With respect to embryogenesis in *C.elegans*, the post-transcriptional regulation of maternal mRNAs is vital to patterning the developing zygote. As an oocyte develops, its chromosomal content becomes transfixed in meiosis until fertilization, preceding transcription of mRNAs inherited by the new organism. Some maternal transcripts that are produced in the immature oocyte are translationally repressed until maturation. This regulation of maturation is crucial to the zygote; mature mRNAs drive the cell cycle and guide the axis patterning and cell fate specification until zygote transcription. Zygotic transcription does not occur until several cell divisions take place, demonstrating the importance of the activation of maternal transcripts through maternal regulatory factors in the formation of the body plan.

This MQP maps the post-transcriptional regulatory system that guides specific axis formation and cell fate specification in *Caenorhabditis elegans*. Various biochemical and molecular genetic methods were implemented to identify the nucleotide sequence specificity and RNA binding specificity of each RNA-binding protein necessary for above mentioned patterning system. Through these experiments, a list of cis-acting regulatory sites will be identified. This research is crucial since it provides an overall map of the post-transcriptional regulatory mechanisms that are necessary for developing zygotes and which are currently not completely understood.

Caenorhabditis elegans

C. elegans is a soil nematode commonly used in a range of different laboratory type experiments including genetics and biology. *C. elegans* are preferred genetic models due to their

short life spans (about 3-4 days) along with them having high fecundity. The species contains two genders: self-fertile hermaphrodites and males which are rare. Both male and female gametes are produced in the same gonad from the same germ line. The life cycle of the *C. elegans* can be divided into the embryonic stage, the four larval stages, and adulthood. In a hermaphrodite, gametogenesis starts during the final larval stage (L4) during which germ cells begin to differentiate into sperm. Between the larval and adulthood stages, oocyte development begins (a process called oogenesis) while sperm production ceases. (Farley & Ryder, 2008)

Oogenesis begins in the distal tip of the gonad of a hermaphrodite worm. (Figure 1) The dividing population of primordial germ cells transition into meiosis where their plasma membranes disappear, forming meiotically arrested nuclei. They then recellularize, therefore generating immature oocytes. These newly developed oocytes remain in meiosis I until they approach an area called the spermatheca (the area contains previously produced spermatocytes), where fertilization occurs. The fertilized oocyte completes meiosis and established anterior-posterior body axis. The area where sperm entry occurs determines the posterior location of the embryo (Goldstein & Hird, 2006) This beginning of body axis determination leads into one of the most crucial aspects to embryo development: embryogenesis.

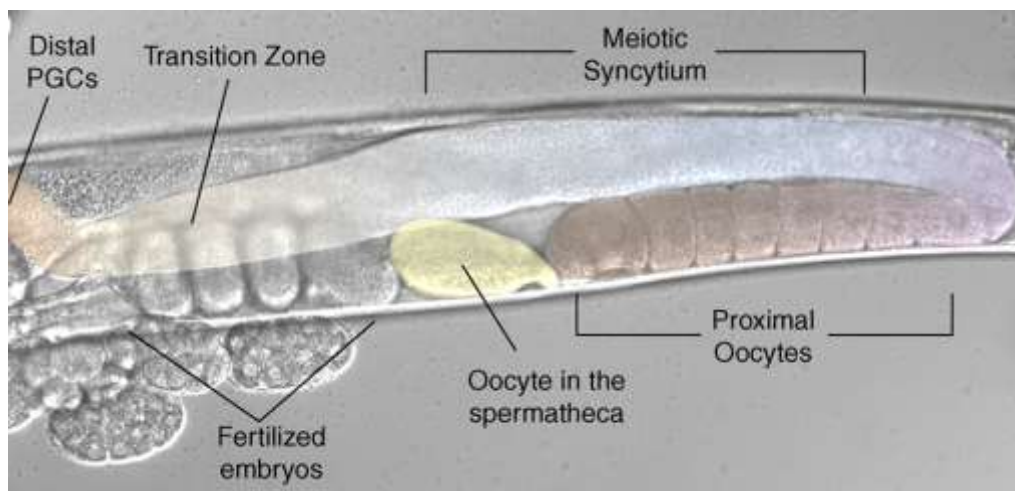


Figure 1: *C. elegans* germline anatomy. (Farley and Ryder, Crit. Rev. Biochem. Mol. Biol., 2008)

Embryogenesis begins with the anterior blastomere (embryo cell) of a two part anterior/posterior cell being the first of six founder cells that form during the beginning cell divisions. (Figure 2) Each of these founder cells must commit themselves to differentiating into a limited set of tissue types. On the contrast, the posterior initial daughter cell is in charge of the entire germline, also called the P lineage (Farley & Ryder, 2008) This division pattern is executed three more times asymmetrically until it undergoes one final symmetric division to produce two primordial germ cells. These germ cells are transcriptionally inactive until larval development. (Farley & Ryder, 2008)

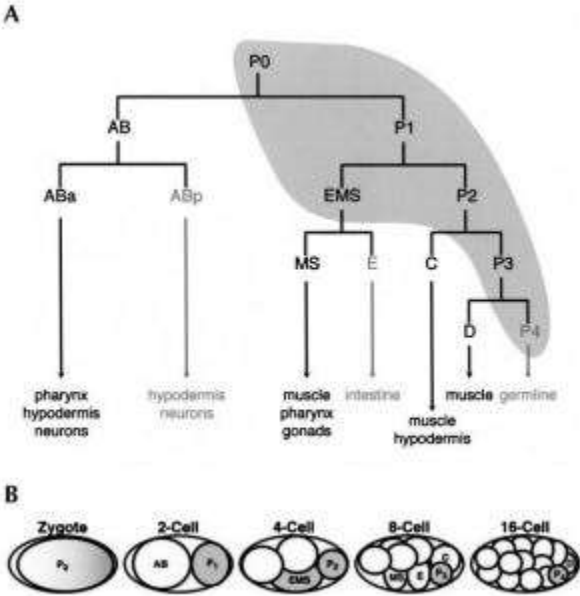


Figure 2: Early Embryogenesis Cell Fate (Farley et al., 2008)

Maternal mRNA and Post-Transcriptional Regulation

It can be specifically seen in *C.elegans* that their lineage is invariant. Maternally expressed genes in particular are responsible for controlling the five rounds of asymmetric

divisions that give rise to the six beginning blastomeres: AB, MS, E, C, D, and P₄. (Landmann et al., 2004) These blastomeres vary in their division rates and progeny as well as individual properties that they hold. ((Sulston et al.,1983).) Only a few of these have been identified to correlate to specific lineages including E (origination of the midgut), P₄ (the germ line), and D (muscles). The complete regulatory pathway of this process is unknown but further research into maternal proteins and regulatory elements indicate a strong tie between the two.

The maternal gamete in *C.elegans* is in charge of cytoplasmic contribution, which contains the maternal proteins and transcripts that guide the patterning of early development that occurs before zygotic transcription. Beginning back at the distal tip of the gonad pre-oogenesis, primordial germ cells (PGCs) produce both the sperm and eggs later required for fertilization. Therefore it is crucial that both commitment to differentiation into haploid gametes and the rate at which daughters of PGC division entering meiosis be highly regulated at the post-transcriptional level.

Since hermaphrodite *C.elegans* produce sperm and egg from the same type of cell (PGCs), it is crucial that the order and timing of production of sperm and eggs be tightly regulated. The switch from spermatogenesis to oogenesis is highly regulated by post-transcriptionally regulated key transcripts that are present in the germline.

Maternal mRNAs are a type of mRNA produced by the maternal genome that is packaged into oocytes to be used in embryogenesis. Cell fate specification is known to occur in the posterior blastomeres during early embryogenesis and is controlled through post-transcriptional regulation by these maternal mRNAs. This regulation is a process that involves RNA-binding proteins interacting with mRNA to control the translation of proteins. Research has identified that mRNA 3' UTRs are the targets for the RNA-binding proteins in germline

progenitors. The 3'UTR is outside of the coding region of mRNA, therefore allowing proteins to bind without any interference from the large ribosome complex. In addition, 3' UTRs aid in capping the coded sequence with a poly-A tail and improving mRNA and protein stability. The entire mechanism of regulation of these proteins is not completely understood but it is confirmed that mRNA-binding proteins and maternal mRNA constructs are important.

An example of one of these transcripts is *fem-3*, which is a transcript produced during the larval stage. FEM-3 protein promotes spermatogenesis and inhibits oogenesis. (Rosenquist & Kimble, 1988) Therefore the transition from spermatocyte to oocyte production requires the silencing of *fem-3* transcripts. (Ahringer & Kimble, 1991) This can be achieved through the binding of one of the proteins FBF-1 or FBF-2 to the point mutation element (a sequence on *fem-3*'s 3'UTR), which in turn represses *fem-3*'s translation and onsets oogenesis. (Zhang et al., 1997) This sequence of events provides a mechanism of reversible silencing of *fem-3* translation. Specific RNA-binding proteins (FBF-1 and FBF-2) control the time of the start of oogenesis by regulating the expression of important genes at a post-transcriptional level.

More specific to the project is the expression of the GLP-1 protein being anti-correlated with POS-1 and GLD-1. GLP-1 aids in the coordination of germline progenitor cell proliferation in addition to anterior fate specification in embryos. It is a key regulator in the switch from mitosis to meiosis in the distal arm of the gonad. (Austin & Kimble, 1987) Both POS-1 and GLD-1 recognize the 3' un-translated region of *glp-1* and have adjacent overlapping binding sites. Therefore POS-1 binding excludes GLD-1 binding. POS-1 and GLD-1 in addition to other RNA-binding proteins (PUF -5/6, PUF-7, and MEX-3) are required for repression of translation at different times in development. GLD-1 represses when germ cells enter meiosis. (Kadyk & Kimble, 1998) PUF-5/6 and PUF-7 act during oogenesis. (Lublin and Evans, 2007) MEX-3

regulates after fertilization. (Pagano et al., 2007) POS-1 along with GLD-1 is needed in the posterior of early developing embryos. (Ogura et al., 2003) Specific areas of *glp-1* have been discovered as regulatory elements: the *glp-1* repression element (GRE) and the *glp-1* de-repression element (GDE) (Marin & Evans, 2003) Mutations on the GRE results in more reporter expression in the posterior area of early embryos, while mutations in the GDE results in little or no reporter expression. (Marin & Evans, 2003) GLD-1 specifically coordinates with the GRE to suggestively repress *glp-1*. (Marin & Evans, 2003) Since the GDE is proximally close to the GRE, it has been suggested that another protein inhibits GLD-1 coordination with the GRE by binding to the GDE. (Marin & Evans, 2003) Overall it is not conclusive whether POS-1 works in conjunction with GLD-1 to repress *glp-1* or if they in turn repress each other, it is necessary to conclude the overall mechanism behind these regulatory proteins because it then in turn will contribute to the knowledge of the overall regulatory pathway required for zygote development.

With such evidence as the potential interactions between maternal regulatory factors like POS-1 and GLD-1, it is crucial to fully understand the complete interactions of protein binding to mRNA since it has been proven to be crucial for regulation. Since it is hypothesized through evidence that there are many factors involved in the regulation of a multitude of 3' UTRs, it may be possible that there are undiscovered factors involved in regulation as well. Understanding the genetic interactions within the post-transcriptional regulatory mechanism of developing zygotes at a molecular level will help properly explain the roles of all maternal regulatory factors as well as much broader implications in the overall study of development.

Library *mos-1* Mediated Single Copy Insertion Method

There are many various methods of transgenic strain generation that have been used over time. The first is called microinjection, which utilizes needles to inject the plasmid that is of

interest right into either the oocytes or meiotic syncytium. Microinjection is a fairly cheap process and is not very time consuming which would make it appear to be an ideal method. Disadvantages do exist such as the likelihood of extra-chromosomal arrays to appear. Another method is called bombardment and is used because it is capable of chromosomal integration without any radiation or chemicals. This process utilizes micro-particles coated in gold with the DNA of interest which are “shot” into the worm at high speeds using a device. This method produces low DNA copy numbers, it is easy to do, and allows potential chromosomal integration. Despite these perks, bombardment is less frequently used due to its cost, the amount of time it takes, and it can potentially cause delocalized expression. (Rieckher et al., 2009)

These two methods have one glaring imperfection in common: random integration. It has been proved that these methods promote chromosomal integration, but the number and location of these integrations is something that cannot be controlled. This makes mutating the target 3' UTRs hard to do and the results unreliable. A method that gives the user control over these factors is called MosSCI (*mosI*-mediated single-copy insertion).

The beginning experiments of this project described here hope to accomplish this deduction of specific binding sites and interactions of regulatory factors on several 3' UTR regions. A process called MosSCI (*MosI*- mediated single copy insertion) will be used to generate single copy transgenic strains that encode GFP (green fluorescent protein) fused to histone H2B with the 3' UTR downstream. The GFP targets H2B to ensure that any expression is restricted to the nucleus. Many 3'UTR genes will be injected to determine expression, creating a library. Each 3' UTR has been chosen specifically since it possesses multiple overlapping binding sites. After injection, those genes expressed will be identified using the reporter and GFP and will be seen in wild type offspring of the injected worms. Specifically we are looking for

asymmetric expression since this indicates the presence of a regulatory pathway in respect to this specific gene.

MosSCI itself is a method to insert a single copy of a transgene into a well-defined location (worm-builder). This method is advantageous because it allows transgenes to be expressed at levels close to endogenous gene expression and these inserts are usually quite stable. In addition, transgene expression is even possible in the germline. To initiate MosSCI, a chromosome must be broken at a specific location through the excision of the heterologous *MosI* transposon when the *MosI* transposase is activated.

A strain must be used that possesses the *MosI* transposon; in this particular experiment the EG6699 strain genome is used. This strain is *unc119(-)* which causes poor coordination in the worms which phenotypically show non-wild-type movement. EG6699 worms also possess the desirable *Mos-I* transposon site at well-defined loci. This allows direct insertion and control over where the transgene will be inserted. To repair this double-stranded DNA break, homologous recombination is employed using specific transgenic templates that were designed with homology arms that match the genomic sequence on the sides of the *MosI* transposon insertion site (Vallin et al., 2012) With this DNA template, the repair process will incorporate DNA from the repair template into the genome.

MosSCI utilizes the method of integration of DNA directly into the worm's chromosome as stated above. With the microinjection technique, we are able to deliver arrays of transgenes to an individual worm along with other components that allow selection and integration. In order to properly integrate, the *MosI* transposon must be present in order for it to be excised by the transposase expressed from a heat-shock promoter. (Bessereau et al., 2001). A site must be specifically chosen for *MosI* so that there are no interferences on any adjacent gene functions

and that there are no enhancers or promoters present that will affect any transgene expression. Generally this site is found on chromosome II, with tail-to-tail orientation at the tTi5605 *MosI* allele. (Frøkjær-Jensen et al., 2008)

In order to see if MosSCI has worked, it is crucial to incorporate a positive selection marker. A positive selection marker selects for an allele that increases fitness. The positive selection marker is incorporated with fluorescence markers to identify if an extra-chromosomal array has formed. An extra-chromosomal array is DNA that exists within the injected worm that is not integrated into the chromosome of the worm. They instead form a “mini-chromosome” which is unstable in meiosis and mitosis resulting in some cells not expressing the transgene. Arrays also contain several copies of DNA which can lead to either over-expression of the gene or toxic effects. The multiple copies of DNA makes it difficult to properly study *C. elegans* since they naturally have the ability to silence repetitive arrays in their germline (Frøkjær-Jensen et al., 2008) This problem occurs in such alternative methods as bombardment and microinjection, but through this single-copy insertion method this problem is overcome. Transgene silencing has been a common problem throughout research of the *C. elegans* germline, in particular research concerning the embryo. (Zeiser et al., 2011) Silencing essentially occurs when repetitive transgene arrays form when DNA is injected into the gonad. In addition to preventing the silencing of extra-chromosomal arrays, MosSCI also prevents transformants from forming with multiple transgene copies, which is unfavorable due to yielding disadvantageous dosage related effects.

The entire injection mix that goes into each worm has positive-selection markers, negative-selection markers, target transgenes and the *Mos I* transposase. As mentioned above, the worms used for injections are unc119(-). In order to recover their wild type movements and

coordination, *unc119(+)* is attached to the target transgenes. This is so that the integration of the transgene will be coupled with the strain of worms recovering the *unc119* gene. This complex is bordered by a left and right homology arm, which both contain approximately 1.4 kb of DNA that is homologous to the genomic DNA adjacent to the *Mos1* transposon. The *unc119(+)*, transgene of interest, and homology arms makes up the entire construct that is intended to integrate upon completion (Fig 3).

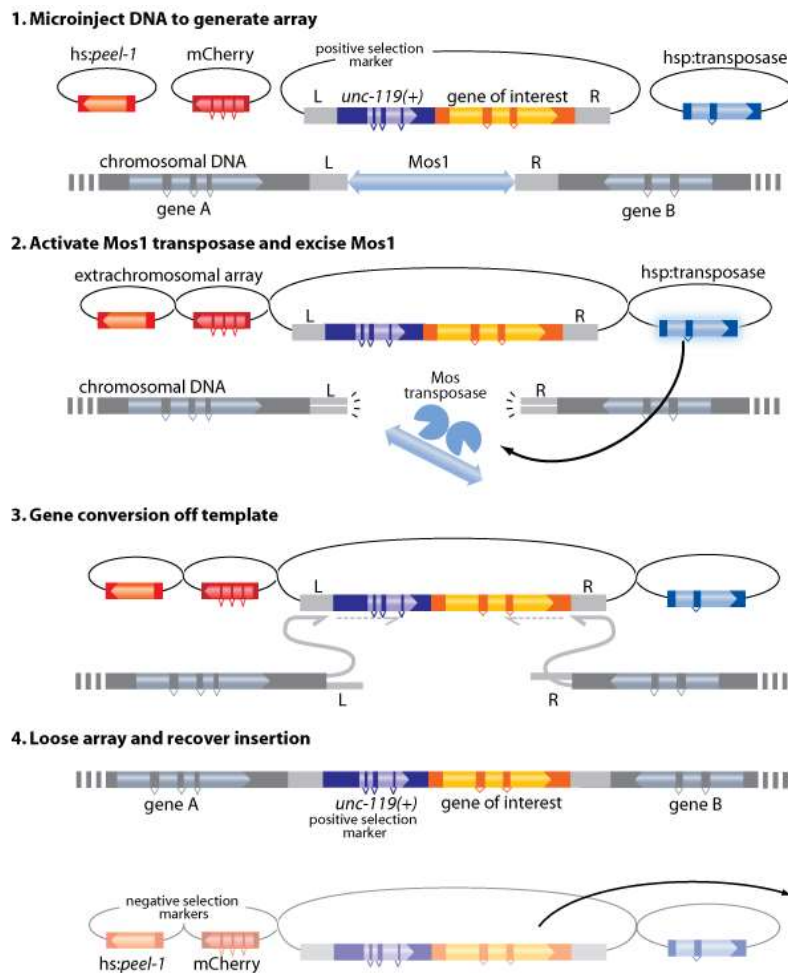


Figure 3: Internal Process of MosSCI (Frøkjær-Jensen et al., 2008)

The negative and positive selection markers are also present in the mix as stated above in order to monitor the loss of any extra-chromosomal arrays that would occur following heat shock. *Peel-1* causes paralysis at 25 degrees Celsius and mCherry targets the pharynx and body wall. Finally, the *Mos1* transposase is in the mix and is expressed as a heat-shock promoter. This allows the excision of *Mos1* and causes the double-stranded DNA break followed by the uptake of the target transgene. (Frøkjær-Jensen et al., 2008)

Overall, MosSCI works most effectively with transgenes that are 7kb or smaller. This produces an efficiency rate of about one successful injection per every twenty worms injected. Strong expression in the germline can be seen with little to no silencing over many generations. (Frøkjær-Jensen et al., 2008) This process of achieving these generations can take about 2-4 weeks to complete and will only yield the one transgenic strain of *C. elegans*. To speed up this process and create a higher yield of transformants, MosSCI must be utilized to produce multiple strains of transgenic worms at one time. The name of this modified approach is Library MosSCI.

Library MosSCI uses the same approach as the general MosSCI method, but instead of inserting an array with just one transgene, a mix of multiples transgene is inserted. In particular for this project, 16 genes were chosen due to them possessing multiple binding sites that overlap. With the MosSCI method, the proper map could be produced in order to pinpoint exact binding sites of each individual transgene. Binding sites were all found within the 3' UTR and were fairly close to one another. This method has the potential to produce multiple transgenic worms through injection containing single-copies of different 3' UTRs that can be used to fully map out post-transcriptionally regulated mechanisms in *C. elegans*.

Materials and Methods

Generating Reporter Constructs

3' UTR amplification and cloning into Gateway compatible vectors

Gateway cloning utilizes an enzyme catalyzed sequence-specific recombination to achieve one step direct insertion into compatible vectors. The 3' UTR of interest was amplified from a wild-type worm's genomic DNA using an Elongase polymerase with the following protocol. The mix included 10 μ l 5 X Elongase buffer B, 5 μ l 2 μ M forward primer, 5 μ l 2 μ M reverse primer, 5 μ l 2 mM dNTPs, 1 μ l 35 ng/ μ l N2 genomic DNA and 23 μ l milliQ water

This mix was flicked and spun down to bring liquid to the bottom of the tube. The reaction was transferred to the thermalcycler and the program was allowed to warm up. Once the temperature on the block reached 95 degrees, 1 μ l of the Elongase enzyme was added to the enzyme mix. The reaction was then allowed to proceed. The program ran as follows: Pre-amplification denaturation: 95 degrees, 2 minutes, Cycling- Denaturation: 95 degrees, 30 seconds, Annealing: 55 degrees, 30 seconds, and Extension: 68 degrees, 2 minutes

This cycle was repeated a total of 35 times. A Qiagen PCR clean-up kit was used to determine the DNA concentration using the Nanodrop to prepare the PCR product for the BP Gateway Reaction.

Plasmid Transformation

Sufficient plasmids must be produced for the injection library. Sixteen 3' UTR constructs (cul-1, mex-3, lin-26, tbb-2, hbl-1, him-14, ets-4, cks-1, cgh-1, set-21, usp-14, mbk-2, atg-4.2, ekk-1, cwn-1, and set-6) along with four extra-chromosomal array constructs [pCFJ601 (Mos-1 transposase), pMA122 (peel-1), pCFJ90 (mCherry pharynx marker) and pCFJ104 (body wall marker)].

Library Assembly

To combine multiple reporter constructs into the library, an equal mass of each construct was mixed together. Sizes of the individual constructs were accounted for in the mix. This library was treated as if it were a single plasmid when making master injection mixes.

Generating Transgenic Worms

MosSCI utilizes a strain of worms that carries a copy of the *Mos1* transposon in the genome. When DNA is injected that encodes the transposase capable of excising that transposon, a genomic double stranded break is formed. Using the transgenic construct flanked by sequences homologous to the double stranded break, a transgene of interest will potentially be inserted into the genome in a single copy at the defined location guided by double stranded repair.

The initial injection strain is uncoordinated and the transgenic construct contained a gene that rescues the phenotype located between the homologous repair regions (adjacent to the transgene). Therefore, rescue of the uncoordinated phenotype was a marker for the presence of a transgene. Uncoordinated worms were injected and their progeny was screened for wild-type rescue. Plates with the rescued worms were then heat-shocked to induce the negative selection marker and surviving wild-type worms likely had a transgene in them.

Master Mix Assembly

Plasmids at the following concentrations were used for MosSCI injections: *Mos1* transposase- 50 ng/ μ l, *peel-1* (heat shock)- 10 ng/ μ l, mCherry (pharynx marker)- 2.5 ng/ μ l, mCherry (body wall marker)- 5 ng/ μ l, and Targeting vector/Library of targeting vectors- 25 ng/ μ l.

The total concentration of DNA was kept low to prevent any stable extrachromosomal array formation. These stable arrays would eventually lead to transgene silencing. Prior to mixing all of the constructs together, they were spun for ten minutes at maximum speed in a microcentrifuge. Around 20-50 μ l of master mix was made at one time, yielding around 12-30 needles' worth.

Preparing Worms for Injection

The most important criterion for a successful integration event is injecting worms of an appropriate age. The injection strain EG6699 is uncoordinated and develops an increasingly severe egg-laying phenotype as it ages. It is critical to inject worms during early adulthood that have about ten embryos in their uterus. Younger worms would not likely survive the injection and older worms would not generate enough progeny to be followed.

At least one week before each planned injection, individual L4 EG6699 worms were picked to 35 mm plates seeded with *Comamonas* (DA1877). This was done every day following that day to create a semi-synchronous population of worms that are ready to inject about a week later. If worms are singled out for multiple consecutive days, it could be ensured that there were always worms available for injection.

Preparing Needles for Injection

At least an hour before injection, needles were pulled and loaded for injection. Kwik-Fil™ capillary tubes were used since they contain an internal filament that speeds up loading. Needles were pulled for microinjection using the Sutter P-97 micropipette puller. Program 50 was chosen since it had been optimized for worm needles. The following settings were used for needle pulling: Pressure=500, Heat= 575, Pull= 45, Velocity= 80 and Delay= 120

While pulling needles, the master mix was spun down at maximum speed in a microcentrifuge for 10 minutes. This prevented particulate matter from being loaded into any needles.

Once the needles were pulled and the master mix had been spun down, the needles were loaded. They were backfilled using an aspirator and a hand drawn needle. Master mix was drawn into the hand drawn needle using the aspirator. The tip of the hand drawn needle was inserted into the back of an injection needle and a few microliters of mix was expelled into the needle. It took between 15 minutes and an hour for the injection mix to settle at the tip of the needle.

When the needles looked loaded, the tips were checked on the dissection scope for any air bubbles. If any air bubbles were present, the needle was discarded. Needles that had no air bubbles were mounted into the microinjector. Once the needle was in the light path, an agar-pad bearing coverslip was prepared. The coverslip agar side up was paced on a 35 mm plate lid under the dissection scope. A drop of halocarbon oil was placed on the coverslip but away from the pad. The oil-bearing coverslip was placed on the injection scope stage with the oil side toward the needle. The needle was gently pushed against the coverslip in order to break the tip of the needle.

When injecting worms, it was best to focus on the gonad under the dissection scope. Once it was in focus, the needle was gently inserted into the gonad. The pump was then pressed and the master mix entered the gonad of the worms, indicated by a swelling of the gonad. Typically only one gonad arm was injected since only one arm is generally visible. Typically in each injection session, 20 to 40 worms were injected. Once injected, the worms recovered at room temperature for an hour. They then were transferred to the 25 degree incubator.

Plasmid Transformation

Sufficient plasmids must be produced for the injection library. Sixteen 3' UTR constructs (*cul-1*, *mex-3*, *lin-26*, *tbb-2*, *hbl-1*, *him-14*, *ets-4*, *cks-1*, *cgh-1*, *set-21*, *usp-14*, *mbk-2*, *atg-4.2*, *ekk-1*, *cwn-1*, and *set-6*) along with four extra-chromosomal array constructs [pCFJ601 (*Mos-1* transposase), pMA122 (*peel-1*), pCFJ90 (*mCherry* pharynx marker) and pCFJ104 (body wall marker)].

Following Injected Worms

After the worms are injected with the array, they were left to propagate at 25 degrees Celsius on 60mm RNAi plates for two days. The worm populations were checked every two days to make sure that starvation had not occurred. Worms were screened for wild type movement, indicating that the *unc-119(+)* gene had been rescued and the injection successful. The plates are then heat shocked for at least an hour at 34 degrees Celsius. After four hours, the negative selection gene (*peel-1*) should be expressed and rescued worms that are still alive indicate proper integration. Chunk half of these plates onto new plates so that the worms must crawl out of the chunk to find food. This allows easy identification of the wild type worms. Follow each strain for a few days, picking wild type worms and re-plating them until you see homozygosity. The strain is now ready to be screened for GFP expression as well as a confirmed insertion through PCR.

PCR verification of insertion

Single worm PCR was used along with a primer that anneals specifically to GFP and a primer that anneals to a region of the genome that is downstream of the insertion site to confirm integration. These primers were BMF69 (forward) and BMF480 (reverse). Worms were initially added to 5 μ l of 30 μ M Tris buffer pH8.8 and 1 mg/mL NEB Proteinase K. The program

NUKEWORM was used (65 degrees for 1 hour and 95 degrees for 15 minutes). While worms are being nuked, another master mix should be prepared for the PCR reaction: 5µl of 10x Pfu Buffer, 5µl of 2µM BMF69, 5µL of 2µM BMF480, 5µL of 2 mM dNTPS, and 24µL of water and multiply each amount of individual product by the number of samples you plan to PCR. Once the worm lysis is completed, 44 µL of the master mix was added to each 5 µl sample of lysed worm. Once the reaction is ready to run, 1 µL Pfu Turbo enzyme was added to each reaction. Run the following program: 95 degrees at 2 minutes, 95 degrees for 30 seconds, 55 degrees for 30 seconds , 68 degrees for 4 minutes, and 68 degrees for 10 minutes. Repeat the cycling steps 35 times total. Next, check the PCR products on a 1X TAE 1% agarose gel. A successful integration should give a single product for each sample. A negative control using an unc119 (-) worm can be used to ensure the PCR was performed correctly.

Sequencing and Fluorescence Imaging

Once it had been confirmed that a transgene has been properly inserted into the strain of worm's genome, the Qiagen PCR cleanup kit was used to prepare the PCR products for sequencing. PCR products were sent out to Elim BioPharm for sequencing. Once the genes were identified with the returning sequence, a fluorescence microscope was used to image individual worms to show expression of GFP and therefore where each 3' UTR was expressed. Strains AD1-8 were identified and put through this process and some were found to be integrated transgenes and some were found to be extrachromosomal arrays that had been missed in the earlier selection testing.

Results

The ultimate goal of this project was to utilize the *Mos1*-mediated single copy insertion method to produce multiple transgenic strains of *C. elegans* in order to accurately map the post-transcriptional regulatory mechanism. In order to accomplish this, a library of 3'UTRs was produced containing multiple possible binding sites.

MosSCI makes use of a particular strain carrying the *Mos1* transposon in its genome. Once DNA that encodes the right transposase is injected, a genomic double strand break is formed when the transposon is excised. A transgenic construct flanked by sequences homologous to the double stranded break aid the potential for a particular transgene of interest to be inserted into the genome in single copy form at a defined location through repair.

Originally, the strain used for injection is uncoordinated (*unc-119*). The transgenic construct engineered for injection contains a gene that is able to rescue the uncoordinated phenotype between the homologous repair regions (which is adjacent to our transgene). Therefore, any rescue of the uncoordinated strain phenotype will mark the presence of the transgene and can be considered a good tool for screening.

The age of the worms being injected is highly crucial for a successful integration event. The strain used (EG6699) is uncoordinated so it develops an increasingly severe egg-laying phenotype as it ages. The prime stage for injection is young adulthood. Typically, a worm with around 10 embryos in its uterus is ideal since worms any younger would most likely not survive the injection and older worms would not generate enough rescued progeny. It was therefore crucial to maintain a fresh stock of worms at all times to not hinder the progression of injections.

Selecting worms for propagation is of vital importance before and after heat shock in order to conserve materials and utilize time. After microinjection and this necessary propagation, the worms were followed for three generations. This was to ensure that the progeny of the injected worms retained their rescue of the *unc119(+)* gene and their wild type movements. The third generation was then screened for the presence of mCherry (pharyngeal and body fluorescence) along with the wild-type movement (defined as sine-wave like movement). Once a majority of the adult worms had the rescued characteristics, this indicated that the transgene was indeed present and was being passed on to progeny through generations. *C. elegans* have the capability of forming extra-chromosomal arrays as mentioned earlier. These typically possess hundreds of copies of your gene of interest and actual expression of the gene is variable from worm to worm. These arrays are silenced in the germline and they are selected against using the two visual screening markers (*mCherry* and *peel-1* for heat shock). Eight lines were followed after the screening markers were implemented. The strains were denoted AD1-8 in order to keep them separate until the proper transgene was identified.

To ensure the presence of 3' UTRs in the worms, a polymerase chain reaction (PCR) test was implemented. This did not necessarily test for chromosomal integration since it could potentially pick up the presence of an extrachromosomal array still present but it did ensure that a 3'UTR was present in the strains. Two primers were used specifically (BMF69 and BMF480) since one anneals to GFP specifically (BMF69) and one anneals to a region of the genome downstream of the insertion site to hopefully confirm integration (BMF480). Additionally, this PCR confirmed that there was only one 3' UTR from the entire library present. Out of the 8 strains, it was confirmed officially through PCR that AD1, AD3, AD4, AD5, and AD7 had transgenes inserted into the genomes while AD2, AD6, and AD8 extrachromosomal arrays.

Multiple PCR tests were performed in order to confirm this and when compiled together, yielded these conclusions. Each PCR was tested along with a negative control [unc-119(-)] to ensure that the PCR worked accurately and that bands expressed could be relied on as evidence of a 3'UTR being present.

Figure 4 is a PCR of the strains AD1, AD2, AD3, and AD4. This PCR used a standard DNA marker as our ladder to visualize where the distance the bands were traveling down the gel. Each PCR that was run was subjected to a gel electrophoresis experiment to visualize the results. As seen in the AD1 lane, there is a clear strong band projected from this PCR. A faint band can be seen underneath, but overall the presence of one band that is stronger than the rest indicates that one gene in the PCR mix was properly amplified through the PCR. Since the primers in the PCR mix are catered to anneal to anything expressing GFP, it is hoped that through proper integration of a transgene that this transgene will be amplified solely in the PCR and shown as a single clear band on the PCR. The lane for AD3 shows a clear strong band as well in a different location, indicating that there is a different transgene inserted into the genome of that line. In addition, another clear strong band is seen in the lane for AD4, indicating yet another transgene inserted into the genome of that line since this band traveled a different distance than both other lines. This PCR was also useful since it showed the lack of a clear strong band for the AD2 lane. This was a red flag for the AD2 line since it showed no indication of there being a transgene present due to no prominent expression of a gene through the PCR. This potentially indicated that AD2 was an extrachromosomal array and therefore the primers had difficulty annealing to a specific transgene.

Figure 5 shows the results of a PCR that tested AD1, AD3, and AD4. This PCR was performed after an initial PCR was done that had shown the presence of a single transgene in

each strain's genome. (Figure 4) AD2 was not tested due to multiple PCR results for it turning up no bands. As seen in Figure 5, each strain was tested in multiple lanes in order to ensure consistency in each loaded sample. Any inconsistencies should indicate that the initial PCR might have been a fluke and results should potentially not be trusted. Figure 5 shows that AD1, AD3, and AD4 had consistent results across each lane tested for each. All bands for each strain resembled each other and were essentially identical to one another. In addition, each lane had a strong, clear band that resembled the bands seen for each respective strain in Figure 4. Since these results matched up to each other so perfectly, it could be concluded that AD1, AD3, and AD4 had properly inserted transgenes that were unique to each other.

Figure 6 shows the PCR results of an entirely new strain, AD7. This strain had been injected later and therefore was tested individually once it had been followed successfully. Since there was only one strain, it was tested in two separate lanes in order to check that it had an identical result to indicate that the PCR was successful. As seen in Figure 6, AD7 has one clear band in both lanes. They are approximately in the same location, showing that there is one integrated transgene and this was solely amplified in the PCR. It could be concluded from this PCR that AD7 has a transgene integrated into its genome.

A PCR was performed to test AD7 along with two other strains that showed homozygosity for wild type traits. AD5 and AD6 had taken longer to express this homozygosity and therefore were tested later. AD7 showed consistent results in Figure 7 when compared to Figure 6. AD7 was tested again to ensure that the results matched and that it again showed single, strong bands. AD5 was also seen to show single strong bands across its three lanes in Figure 7. This indicates that AD5 could potentially have a transgene inserted and that it should be checked with sequencing. AD6 was seen to not have any clear strong bands that stood out in its four lanes

tested in Figure 7. These results were similar to those seen in Figure 4 with AD2, indicating that AD6 potentially was an extrachromosomal array.

A final strain was identified through selection to express homozygosity for wild type traits, indicating a rescue. AD8 was subjected to PCR alone and the results were tested using gel electrophoresis. Figure 8 shows the four lanes tested with AD8. It can be seen that there is similar expression to AD2 and AD6 with multiple bands appearing in each lane. This indicates that the primers used in PCR had annealed to multiple locations and therefore the PCR had amplified multiple genes. Therefore it can be concluded from Figure 8 that AD8 also possessed an extrachromosomal array. This fact in combination with AD2 and AD6 having extrachromosomal array shows that with library *mos-1* mediated single copy insertion, there is still potential for extrachromosomal arrays to pass all of the selection marker tests and appear to carry a transgene. The selection marker portion of MosSCI is not fool-proof but it does aid in cutting down both time and resources that could be implemented on numerous strains that would yield extrachromosomal arrays. Knowing that these three strains most likely expressed extrachromosomal arrays, these three strains were still sent to sequencing just in case there was an error in the PCR and a gene actually was expressed. All three strains that appeared as extrachromosomal arrays came back negative from sequencing, therefore no transgene was inserted in AD2, AD6, and AD8.

A final PCR was performed with these four strains that were going to be imaged. AD5 was sent for sequencing but due to time constraints, could not be propagated and imaged in time. Figure 9 shows the results of AD1, AD3, AD4, and AD7 compared to one another. The ladder in Figure 9 is faint due to the fact that it was diluted because there was not enough on hand to fill the lane. It can still be seen and therefore the Ethidium Bromide properly annealed to the loading

dye. AD1 shows multiple bands with no strong singular band being prominent against the others. This indicates that the PCR performed on this strain did not work properly and there was potentially some contamination. Due to the fact that the other two PCR tests showed positive results for AD1 expressing a single transgene along with positive sequencing returning for AD1, it was concluded that this PCR for this strain was due to human error. AD3, AD4, and AD7 showed the same expression as previous PCRs in Figure 9 (AD7 shows only a partial band due to a gel forming error). This PCR further indicated that AD3, AD4, and AD7 in particular were of interest for sequencing.

From these PCR results, it can be seen that there were definitive bands overall for AD1, AD3, AD4, AD5, and AD7 indicating that the GFP had annealed to one 3' UTR and the primers from the PCR picked up on the GFP presence and amplified the one 3' UTR accurately. At this point, the PCR products from each positive strain were sent for DNA sequencing in order to identify each strain's identification. Out of the library injected, five different 3' UTRs were retrieved from the sequence DNA; AD1: *atg-4.3*, AD3: *hbl-1*, AD4: *mex-3*, AD5: *usp-14*, and AD7: *lin-26*. With these 3' UTRs sequenced, the next step was to see where the genes were expressed in the *c. elegans* germline.

In order to see where the GFP fluoresced, a fluorescence microscope was used. Worms were picked for imaging based on their ages. Young adulthood worms were targeted since they generally had a single line of embryos formed at this life stage and this allowed us to see any presence of fluorescence as these embryos formed. If fluorescence was not seen at this life stage, other life stages such as larval stage worms were also imaged to see if the gene was being expressed at an alternative life stage. The expression patterns were identified with this imaging and compared to any known patterns observed in previous research. Initially, Differential

Interference Contrast (DIC) imaging was employed to aid in focusing on areas of interest (ex. germline loop). Once a good focus was found and the germline was clearly visible, a GFP fluorescence image was taken in order to see any presence of GFP. Each strain was imaged to obtain over 200 images each in order capture as many images of fluorescence as possible. These images were compared to each other to ensure consistency of expression over each strain. All strains were imaged except AD5 due to time constraints. Below are the results from each strain's imaging session along with a discussion of what each expression pattern indicates.

AD1: *atg-4.2*

Figure 10 focuses on the meiotic syncytium of an AD1 adult worm along with the germline loop where the oocytes become more pronounced. These are stages of embryogenesis where it as hoped that there would be expression of the gene.

Figure 11 confirms that there is no expression of GFP other than basic gut fluorescence in this AD1 adult worm. This gut fluorescence was seen in every AD1 worm screened and is attributed to the food the worms eat. This image shows no other expression of GFP, indicating no expression of the corresponding 3' UTR and therefore no activity at each respective stage. Worms that were one life stage older and one life stage younger were screened as well with the same projected results. Out of around 100 + images taken, only gut fluorescence was observed in the AD1 strain. This does not exactly indicate a lack of this 3' UTR functioning in embryogenesis, it just does not indicated any potential expression pattern.

When looking at past research, *atg-4.2* is one of the lesser studied 3' UTRs. No observed expression pattern has been discovered and therefore could not have been compared to our expression pattern had we yielded one. Therefore we could not pinpoint if any other life stage

could yield better results. The 3'UTR could have not been integrated completely into that strain's genome.

In other studies, *atg-4.2* has been studied along with its correlation to *C. elegans*' embryogenesis. *C. elegans* contain two different *atg-4* (a cysteine protease) homologs: *atg-4.1* and *atg-4.2*. One study showed that genetic mutations in *atg-4.1* caused defective degradation of many protein aggregates during embryogenesis, but genetic mutations in *atg-4.2* maintained normal levels of removing these substrates. (Wu et al., 2012) This indicates that *atg-4.2* does play a part in embryogenesis and therefore it should continuously be tested in genetic experiments such as this to reveal its full function and expression in the germline.

AD3: *hbl-1*

Figure 12 focuses on the more developed oocytes of an AD3 adult worm as they are entering into the spermatheca along with newly fertilized embryos. Around 200 + images were taken of this strain looking at multiple ages along with multiple steps embryogenesis (including earlier in the loop). The best expression pattern was seen in later developed oocytes and embryos after leaving the spermatheca. There was no observed expression in the spermatheca for AD3 worms.

As can be seen in Figure 13, there is increasingly stronger GFP fluorescence seen in the developing oocytes of the AD3 adult worm imaged. Some early expression is seen in the beginning of the germline loop but is more prominent as the oocytes line the gonad wall in single file and become larger and more pronounced. This expression leads up to the spermatheca (the blank space between expression) where there is no observed expression. After the oocytes are fertilized, expression returns and is strongest before the embryos begin to divide. The expression

eventually begins to fade out as the embryos go through more division cycles and it eventually disappears.

The HBL-1 protein has the most intense signal in fertilized embryos as confirmed in the GFP image. This signal decreases in L1s and is virtually absent in the hypodermis of an L3 animal. (Lin et al., 2003) Strongest fluorescence in the GFP image is seen as expected in fertilized embryos after passing through the spermatheca. Therefore results obtained in this experiment were confirmed with existing results. Since there was expression of the *hbl-1* gene in stages of embryogenesis, it was important to find out what information was already known about it.

The *hbl-1* (hunchback-like) gene in *C. elegans* is a well-known transcription factor primarily responsible for temporal patterning. Proper temporal development is especially important in *C. elegans* since it ensures that a developing organism adopts the correct positional outcome. It also can be considered a probable target for microRNA regulation, in particular its 3' UTR. This is shown specifically through complementation found between the *hbl-1* 3' UTR and microRNAs known for regulation. (Lin et al., 2003)

MicroRNAs are single stranded RNA molecules known for regulating such various processes as development, metabolism, cell differentiation, etc. (Kloosterman & Plasterk, 2006.) They directly post-transcriptionally regulate messenger RNA (mRNA) targets by binding through complementarity to their 3' untranslated regions. One specific miRNA known to interact with HBL-1 is *let-7*. (Rouch & Slack, 2009)

HBL-1 is also known to downregulate the transcription of *let-7* (an important regulator of developmental timing and cell differentiation). Therefore HBL-1 regulates developmental timing and can inhibit adult development in the larval stages. The inhibition of *let-7* allows

proper development of the larval stages and maintains proper cell fates. This type of interaction demonstrates a negative feedback loop mechanism, which is a self-regulating system that detects changes in the system and produces the proper response to counter-acts the change.

AD4: *mex-3*

Figure 14 captures not only every stage of developing oocytes for an AD4 adult worm but also many cell divisions of fertilized embryos. This worm was a slightly older adult that was farther along in its reproductive process.

As seen in Figure 15, there is strong *mex-3* expression throughout the later development of the oocytes in the adult AD4 worm and also in the dividing fertilized egg. The *Mex-3* protein is known to express in the syncytial core of the gonad arm and distribute uniformly in oocytes and early 1-cell embryos. Once the first division occurs, MEX-3 protein becomes more abundant in the anterior AB cell. After the 4-cell stage, *mex-3* mRNA expression disappears.

(Wormbase)

Mex-3 is another maternally-supplied factor that controls RNA metabolism of transcripts encoding critical cell fate determinants. (Pagano et al., 2007) It plays key roles in the renewal of totipotent stem cells in the germline and cellular differentiation. Currently, nucleotide sequence specificity and actual mRNA recognition are currently not completely understood. Since *mex-3* contains conserved RNA-binding domains, it most likely has a role in development at the posttranscriptional level. (Pagano et al., 2007) It is shown that *mex-3* may negatively regulate spatial and temporal development of *pal-1* and *nos-2* through their 3' UTR. (Jadhav & Subramaniam, 2008) PAL-1 is required to specify the posterior blastomere. NOS-2 is necessary for proper development of primordial germ cells.

AD-7: *lin-26*

Figure 16 shows the DIC image of two AD7 strain larval stage 4 worms. It highlights both of their gonad arms where oocytes develop before fertilization. Figure 17 shows the GFP image of the same two worms. It can be seen that there is a lot of basic gut fluorescence, making it hard to see other fluorescence. When looking closely at the gonad arm specifically, early oocytes (nuclei) can be seen in clusters before the entire the germline loop when they begin to form a single line. Some fluorescence can be seen after the oocytes pass through the loop, indicating that there is also some expression in the later developed oocytes. The fluorescence quickly fades and disappears as the oocytes enter the spermatheca and become fertilized. Therefore there is no seen expression in the fertilized embryos. Expression does not begin until after the distal tip, where the germ cell precursors reside.

The Lin-26 protein has been detected in all cells of the somatic gonad except in the distal tip cells. Expression became weaker as cell division occurred and eventually vanishes. When looking at Figure 17, it can be seen that there is expression throughout the gonad, but it disappears once it reaches the distal tip (near cell division).

Lin-26 is a gene that is required in *C.elegans* for proper differentiation of epithelial cells in the gonad. It is typically expressed as early as the embryo stage of growth and remains through a worm's development. Typically, *lin-26* has been known to be expressed by various cell types in *C.elegans* including epithelial cells, gonad, and uterus in particular interest. It has been seen in particularly the epithelial cells that in the presence of *lin-26*, the cells tend to degenerate indicating that *lin-26* is needed for their differentiation. (Labouesse et al., 1996)

Many cells that have been seen to express *lin-26* originate from the AB blastomere, the C blastomere or from the MS blastomere. The onset of *lin-26* being expressed depends on the

specific tissue or cell type. (Labouesse et al.,1996.) The ectopic expression of LIN-26 during early gastrulation allows blastomeres to be transformed into epithelial-like cells. (Quintin et al., 2001)

Discussion

Germ-cell lineage in any organism dictates the conservation of all genetic material required as important information that must be passed on from generation to generation. In early embryogenesis in *C. elegans*, germ cells are distinctly specified from the somatic cells. Due to various controlled mechanisms directed by protein mRNA interactions, each cell division that occurs has specific steps that occur in order to maintain the germline in a specific pattern that is required for an organism's survival. It is therefore crucial to understand the exact controlling mechanisms that go into the process in order to fully understand the specification of the somatic cells. *C. elegans* is a widely used organism in many forms of genetic research, therefore understanding these mechanisms is important in order to fully understand how the organism is developed. Many factors seen in embryogenesis of *C. elegans* are also seen in other organisms. This fact allows a relatively easy study of the germline specification and development of other organisms, specifically in the understanding of other eukaryotic systems. With this overlap along with the importance of *C. elegans*, studying the regulatory pathway of the organism's specification has many potential applications across the life science fields.

Key factors in this regulation are the modifications that occur in order to keep the process running smoothly and at a pace that is specified internally by the organism. Examples of these modifications include silencing, expression of certain genes, repression, and activation. While a large portion of germline specification is modified by genetics, an unknown amount is also mediated by these types of modifications based on given conditions inside an individual organism. This study was aimed at observing potential interactions between the 3' UTRs of several mRNAs and RNA-binding proteins. It is currently unknown what governs the rate of these interactions and when certain regulatory genes are turned on or off in embryogenesis.

Experiments performed in this study were implemented in order to see the potential in a specific modified process: Library mos-1 mediated single copy insertion. Results obtained from the experiments were compared to existing results to ensure accuracy in the insertion of a transgene in a strain of worms' genome. With the proper insertion, imaging allowed the viewing of where the inserted transgene was expressed within the worm's embryogenesis process.

Multiple different transgene strains of *C.elegans* were produced that contained a single copy of a 3' UTR at the defined locus of the chromosome using the mos-1 mediated single copy insertion method. The method was a modified version through the preparation of the injection mixture, where sixteen transgenes were combined rather than just one. This library method yielded five different transgenic strains: (*atg-4.2*, *hbl-1*, *mex-3*, *usp-14*, and *lin-26*). The time it took to generate these five transgenic strains was about the same time it would have taken for the original non-library MosSCI to generate a single strain. The strains possessed a single copy of the 3'UTR fully integrated in the respected chromosome and germline GFP expression was obtained. These GFP images indicated various locations where potential RNA-binding proteins may interact with and bind.

With the success of actual germline expression of the GFP, it can be confirmed that Library MosSCI is an effective method for inserting transgenes. Despite its success, the process can undergo further optimization to obtain stronger germline expression along with more integrated strains per injection. An important factor in the success of an injection is the proper propagation and selection of an age appropriate worm for injection. By selecting a fit worm of the proper age, the chances of proper insertion could be increased. This method could further be utilized to include hundreds of transgenes in the injection mix by just lowering the plasmid concentration within the mix.

Overall, the MosSCI injection method was a success in utilizing time and resources in the generation of transgenic strains. With more time, specific mRNA-binding proteins can be compared against each mRNA binding domains to aid in the creation of a regulatory map. Library MosSCI with proper use could become a primary method in the generation of transgenic *C.elegans* strains and could potentially be branched into other animals. This method overall could essentially be highly useful in understanding the mechanics of translational regulation.

Figures

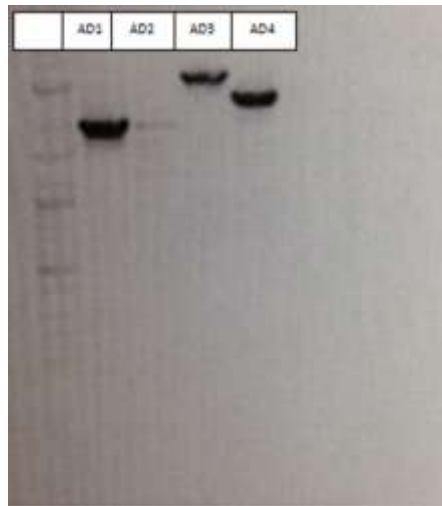


Figure 4: PCR of strains AD1, AD2, AD3, and AD4 to test for single 3' UTR

Single worm PCR was used along with the primers BMF69 (forward) and BMF480 (reverse). Worms were initially added to 5 μ l of 30 μ M Tris buffer pH8.8 and 1 mg/mL NEB Proteinase K for worm lysis. A mix of 5 μ l of 10x Pfu Buffer, 5 μ l of 2 μ M BMF69, 5 μ L of 2 μ M BMF480, 5 μ L of 2 mM dNTPS, and 24 μ L of water was used for the PCR. Next, the PCR products were checked on a 1X TAE 1% agarose gel. A successful integration should give a single product for each sample. A negative control using an uncl119 (-) worm can be used to ensure the PCR was performed correctly. Lanes for AD1, AD3 and AD4 showed single copy insertion of a transgene.

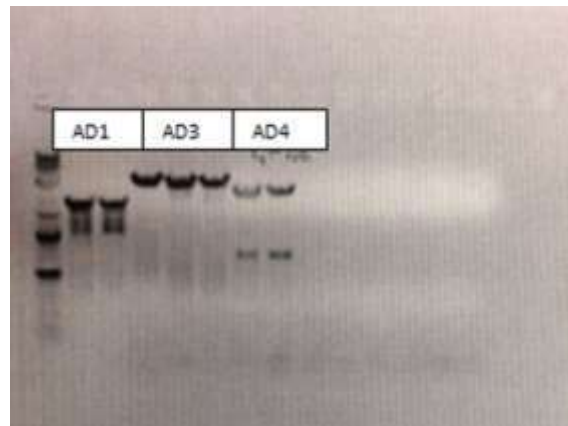


Figure 5: PCR of strains AD1, AD3, and AD4 to test for a single 3' UTR

Single worm PCR was used along with the primers BMF69 (forward) and BMF480 (reverse). Worms were initially added to 5 μ l of 30 μ M Tris buffer pH8.8 and 1 mg/mL NEB Proteinase K for worm lysis. A mix of 5 μ l of 10x Pfu Buffer, 5 μ l of 2 μ M BMF69, 5 μ L of 2 μ M BMF480, 5 μ L of 2 mM dNTPS, and 24 μ L of water was used for the PCR. Next, the PCR products were checked on a 1X TAE 1% agarose gel. A successful integration should give a single product for each sample. A negative control using an uncl119 (-) worm can be used to ensure the PCR was performed correctly. Lanes for AD1, AD3, and AD4 showed single copy insertion of a transgene.

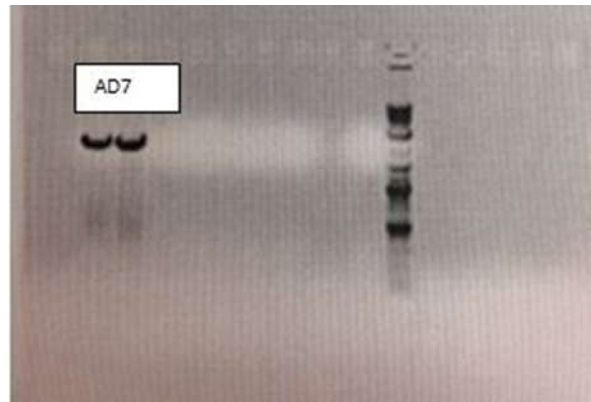


Figure 6: PCR of strain AD7 to test for a single 3' UTR

Single worm PCR was used along with the primers BMF69 (forward) and BMF480 (reverse). Worms were initially added to 5 μ l of 30 μ M Tris buffer pH8.8 and 1 mg/mL NEB Proteinase K for worm lysis. A mix of 5 μ l of 10x Pfu Buffer, 5 μ l of 2 μ M BMF69, 5 μ L of 2 μ M BMF480, 5 μ L of 2 mM dNTPS, and 24 μ L of water was used for the PCR. Next, the PCR products were checked on a 1X TAE 1% agarose gel. A successful integration should give a single product for each sample. A negative control using an unc119 (-) worm can be used to ensure the PCR was performed correctly. The lanes for AD7 showed single copy insertion of a transgene.

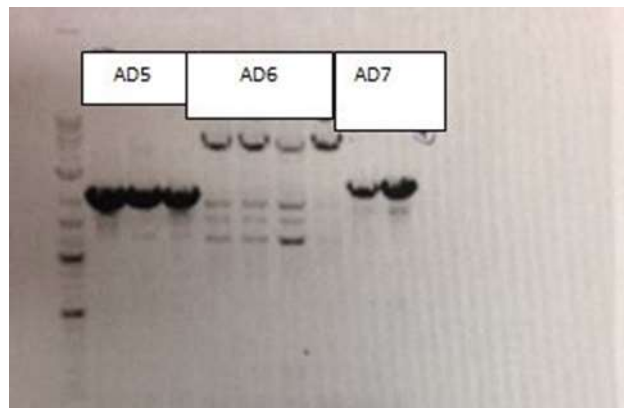


Figure 7: PCR of AD5, AD6, and AD7 for a single 3' UTR

Single worm PCR was used along with the primers BMF69 (forward) and BMF480 (reverse). Worms were initially added to 5 μ l of 30 μ M Tris buffer pH8.8 and 1 mg/mL NEB Proteinase K for worm lysis. A mix of 5 μ l of 10x Pfu Buffer, 5 μ l of 2 μ M BMF69, 5 μ L of 2 μ M BMF480, 5 μ L of 2 mM dNTPS, and 24 μ L of water was used for the PCR. Next, the PCR products were checked on a 1X TAE 1% agarose gel. A successful integration should give a single product for each sample. A negative control using an unc119 (-) worm can be used to ensure the PCR was performed correctly. Lanes for AD5 and AD7 showed insertion of a single copy of a transgene.

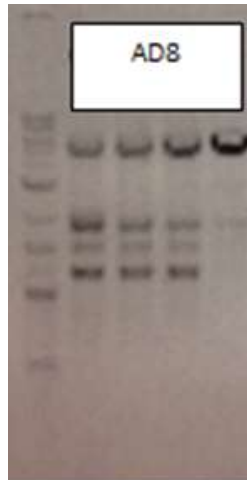


Figure 8: PCR of AD8 strain for a single 3' UTR

Single worm PCR was used along with the primers BMF69 (forward) and BMF480 (reverse). Worms were initially added to 5 μ l of 30 μ M Tris buffer pH8.8 and 1 mg/mL NEB Proteinase K for worm lysis. A mix of 5 μ l of 10x Pfu Buffer, 5 μ l of 2 μ M BMF69, 5 μ L of 2 μ M BMF480, 5 μ L of 2 mM dNTPS, and 24 μ L of water was used for the PCR. Next, the PCR products were checked on a 1X TAE 1% agarose gel. A successful integration should give a single product for each sample. A negative control using an unc119 (-) worm can be used to ensure the PCR was performed correctly. Lanes for AD8 show no single copy insertion of a transgene.

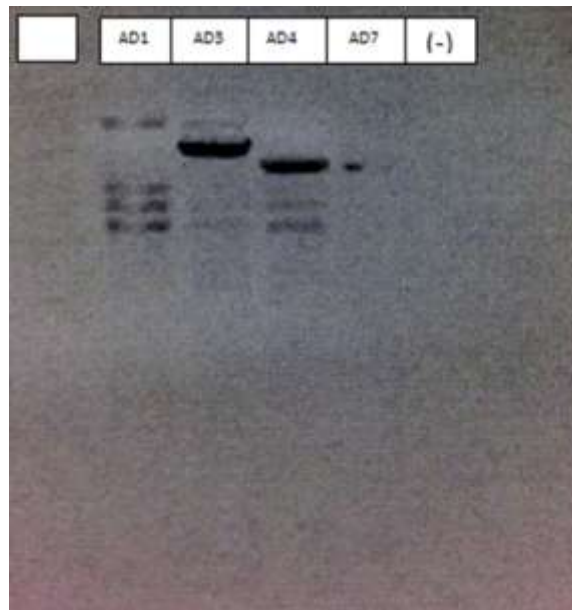


Figure 9: PCR of AD1, AD3, AD4, and AD7 strains for a single 3' UTR

Single worm PCR was used along with the primers BMF69 (forward) and BMF480 (reverse). Worms were initially added to 5 μ l of 30 μ M Tris buffer pH8.8 and 1 mg/mL NEB Proteinase K for worm lysis. A mix of 5 μ l of 10x Pfu Buffer, 5 μ l of 2 μ M BMF69, 5 μ L of 2 μ M BMF480, 5 μ L of 2 mM dNTPS, and 24 μ L of water was used for the PCR. Next, the PCR products were checked on a 1X TAE 1% agarose gel. A successful integration should give a single product for each sample. A negative control using an unc119 (-) worm can be used to ensure the PCR was performed correctly. Lanes for AD3, AD4, and AD7 show the confirmation that a single copy of a transgene was inserted. The lane for AD1 shows the results of human error.

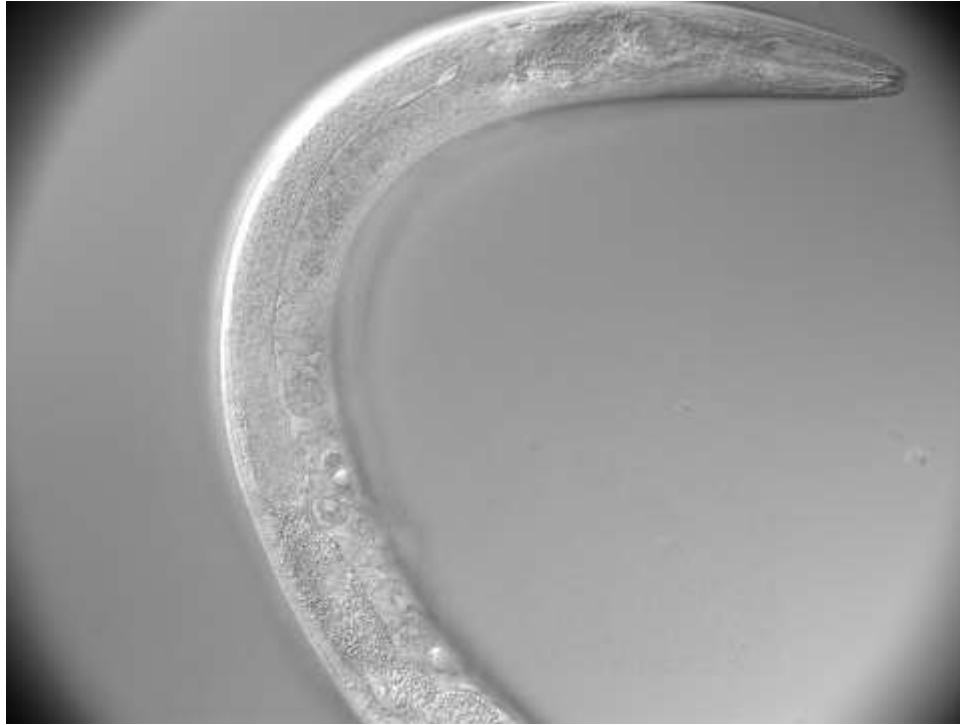


Figure 10: DIC Image of AD1 adult stage worm (atg-4.2)

This DIC image focuses on the meiotic syncytium of an AD1 adult worm along with the germline loop where the oocytes become more pronounced. These are stages of embryogenesis where it is hoped that there would be expression of the gene.

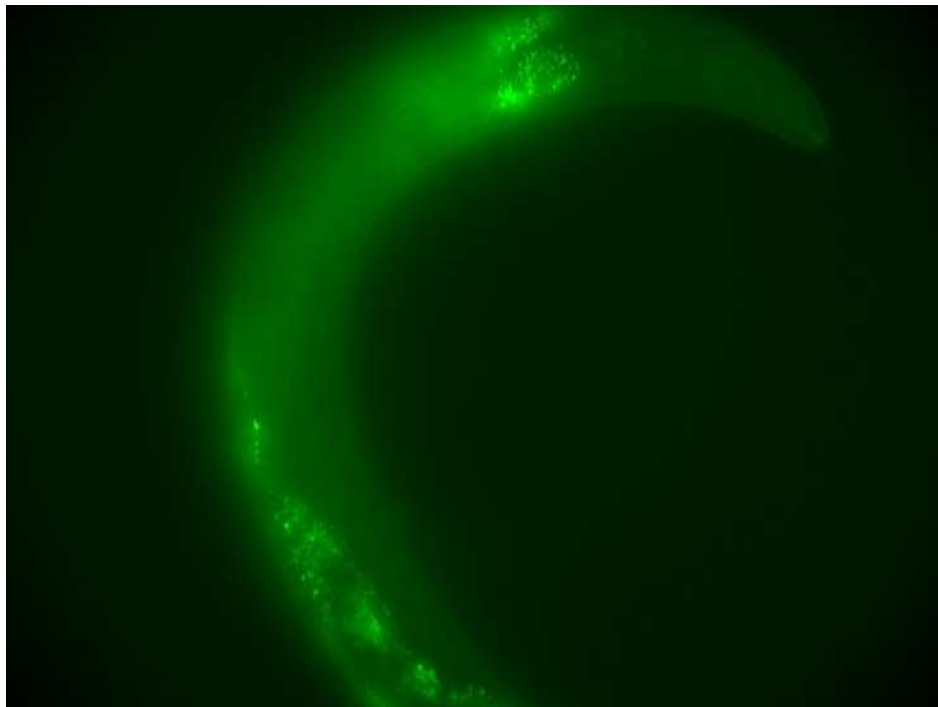


Figure 11: GFP Image of AD1 adult worm (atg-4.2)

Figure 11 confirms that there is no expression of GFP other than basic gut fluorescence in this AD1 adult worm. This gut fluorescence was seen in every AD1 worm screened and is attributed to the food the worms eat. This image shows no other expression of GFP, indicating no expression of the corresponding 3' UTR and therefore no activity at each respective stage.



Figure 12: DIC image of AD3 adult worm (hbl-1)

Figure 12 focuses on the more developed oocytes of an AD3 adult worm as they are entering into the spermatheca along with newly fertilized embryos.

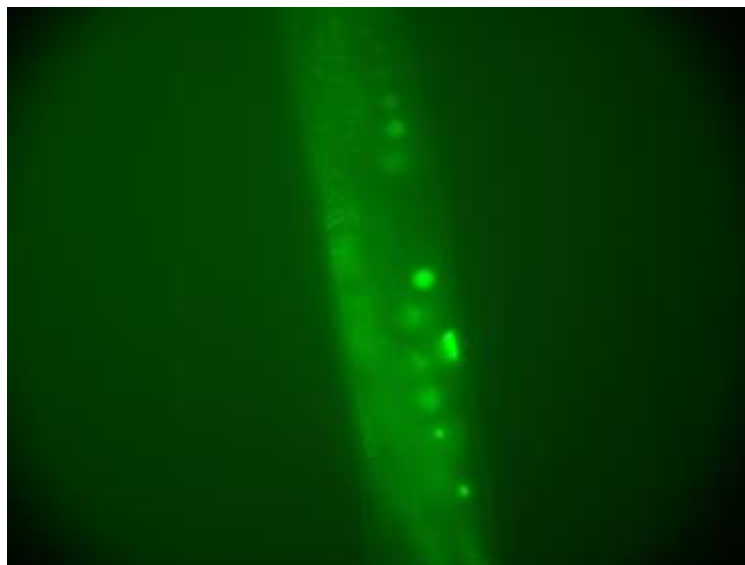


Figure 13: DIC image of AD3 adult worm (hbl-1)

As can be seen in Figure 13, there is increasingly stronger GFP fluorescence seen in the developing oocytes of the AD3 adult worm imaged. Some early expression is seen in the beginning of the germline loop but is more prominent as the oocytes line the gonad wall in single file and become larger and more pronounced. This expression leads up to the spermatheca (the blank space between expression) where there is no observed expression. After the oocytes are fertilized, expression returns and is strongest before the embryos begin to divide. The expression eventually begins to fade out as the embryos go through more division cycles and it eventually disappears.

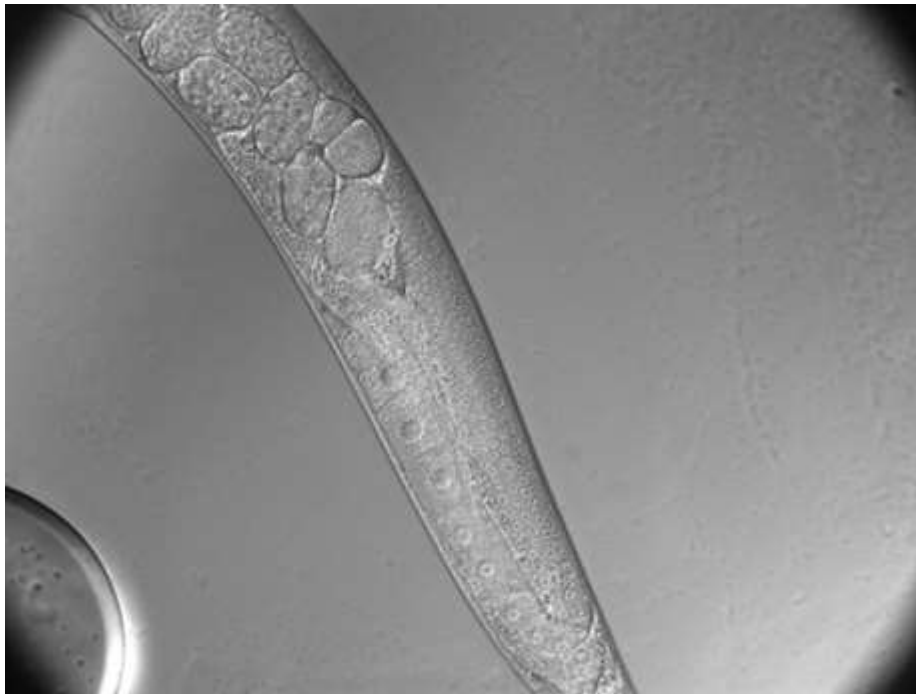


Figure 14: DIC image of AD5 adult (mex-3)

Figure 14 captures not only every stage of developing oocytes for an AD4 adult worm but also many cell divisions of fertilized embryos. This worm was a slightly older adult that was farther along in its reproductive process.

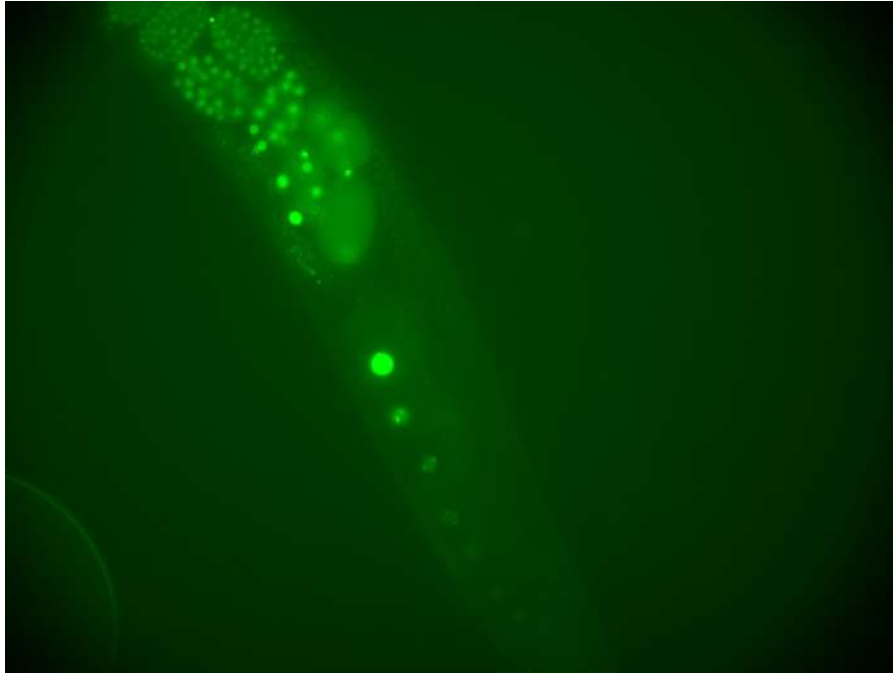


Figure 15: GFP image of AD4 adult worm (*mex-3*)

As seen in Figure 15, there is strong *mex-3* expression throughout the later development of the oocytes in the adult AD4 worm and also in the dividing fertilized egg.



Figure 16: DIC image of AD7 L4 (*lin-26*)

Figure 16 shows the DIC image of two AD7 strain larval stage 4 worms. It highlights both of their gonad arms where oocytes develop before fertilization.

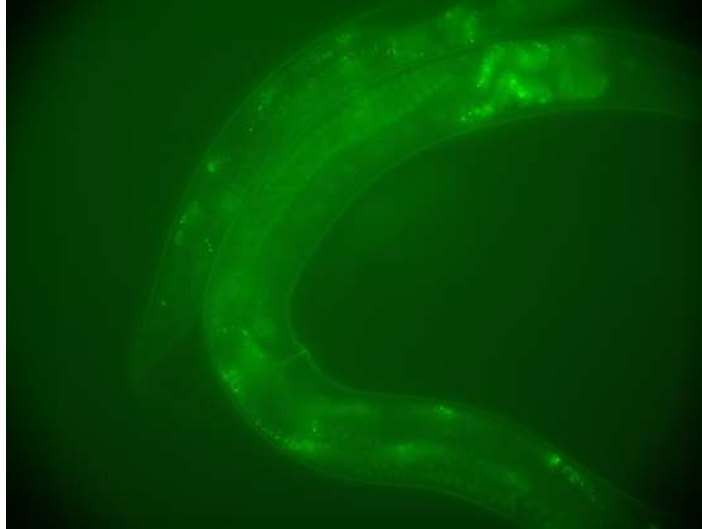


Figure 17: GFP image of AD7 L4 stage worms (*lin-26*)

Figure 17 shows the GFP image of the same two worms. It can be seen that there is a lot of basic gut fluorescence, making it hard to see other fluorescence. When looking closely at the gonad arm specifically, early oocytes (nuclei) can be seen in clusters before the entire the germline loop when they begin to form a single line. Some fluorescence can be seen after the oocytes pass through the loop, indicating that there is also some expression in the later developed oocytes. The fluorescence quickly fades and disappears as the oocytes enter the spermatheca and become fertilized. Therefore there is no seen expression in the fertilized embryos. Expression does not begin until after the distal tip, where the germ cell precursors reside.

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