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Isolation and Characterization of the Y32G9A.8 Promoter in *C. elegans*

by

Rebecca Joy Schlisner

Under the Direction of W. William Walthall

ABSTRACT

The over-expression of Down syndrome cell adhesion molecules (DSCAMs) is partially responsible for the mental retardation associated with Down syndrome. Previous work in our lab showed that a DSCAM homolog in *C. elegans,* Y32G9A.8*,* is expressed at all developmental stages and appears to be crucial for survival. In an effort to map the expression pattern, I used the Genome Sciences Centre's primer design program (http://elegans.bcgsc.bc.ca/gfp_primers/) to design a GFP promoter fusion product that was used to monitor gene expression. The results indicate that Y32G9A.8 is expressed in the animal's gut, suggesting that it may function in the worm's innate immune response. I also designed a primer set to amplify the Y32G9A.8 transcript. RT-PCR of the entire Y32G9A.8 coding region resulted in a single product; there appears to be no alternative splicing. Although this gene shows homology to other N-CAMS, results indicate that this gene may function in the innate immune system of *C. elegans*.

INDEX WORDS: *C. elegans,* DSCAM, GFP, innate immunity

ISOLATION AND CHARACTERIZATION OF THE Y32G9A.8 PROMOTER IN *Caenorhabditis elegans*

by

Rebecca Joy Schlisner

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2006

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Rebecca Joy Schlisner
2006

ISOLATION AND CHARACTERIZATION OF THE Y32G9A.8 PROMOTER IN

Caenorhabditis elegans

by

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Office of Graduate Studies
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December 2006

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LIST OF ABBREVIATIONS

CAM: Cell Adhesion Molecule

N-CAM: Neural Cell Adhesion Molecule

DSCAM: Down Syndrome Cell Adhesion Molecule

ABF: Antibacterial Factor

DAF: Abnormal Dauer Formation

IGF: Insulin Growth Factor

TIR: Toll/interleukin-1 Receptor

IgCAM: Immunoglobulin Cell Adhesion Molecule

RT-PCR: Reverse Transcription Polymerase Chain Reaction

RNAi: RNA interference

GFP: Green Fluorescent Protein

PCR: Polymerase Chain Reaction

DEPC: Diethyl Pyrocarbonate

DS: Down Syndrome

INTRODUCTION

C. elegans is a free-living, non-parasitic soil nematode that is commonly used in the laboratory for genetic study and manipulation. It is inexpensive and easy to maintain in the laboratory, feeding on bacteria, and large numbers of animals can be grown on a single Petri dish. It is small (about 1 mm in length) and transparent for ease of manipulation and observation. *C. elegans* has five pairs of autosomes and one pair of sex chromosomes; it has two sexes, hermaphrodites (XX) and males (XO). Hermaphrodites can self-fertilize and are quite prolific--one hermaphrodite can produce over 300 progeny through self-fertilization. In nature, hermaphrodites are the most common sex, with males being spontaneously produced about 1/1000 of the time due to non-disjunction of the sex chromosomes. In addition, *C. elegans* has a short life cycle. From egg to egg takes about 3 days, and its life span is around 2 to 3 weeks under appropriate conditions. *C. elegans*' short reproductive cycle makes it an ideal organism for genetic crosses and manipulations (1).

C. elegans has a simple body structure and a small number of cells: 959 somatic cells including 302 neurons. Because the body of *C. elegans* is transparent, it is easy to visualize individual cells using reporter genes that produce a visible gene product in the animal. The nervous system has been studied extensively, and the complete cell lineage of the nervous system, as well as the other somatic cells, can be mapped from fertilization (2). There appears to be no variation between animals in the branching structures of the neurons and the connections they make; this feature makes *C. elegans* an ideal model system for studying neural cell development because all animals show the same

patterning of neurons. Classification of the neurons is based on cell morphology and synapse connectivity, and there are 118 distinct neuronal classes in *C. elegans* (2).

Nervous System Development

Early in development of the nervous system, it is critical that neurons are directed as to which way they should migrate. The extracellular surroundings of neurons play a key role in guiding neural cell migration. Axonal outgrowth is directed by means of mechanical guidance in which cell adhesion plays an important role in building neuronal connections (3). Close contact between neighboring cells, i.e. cell adhesion, helps neurons form precise, highly specific circuits by directing axons towards their target cells. Proteins found on the surface of developing neural cells, cell adhesion molecules (CAMS), help form adhesive molecular bonds between adjacent cells. These highly specific bonds help to create the highly structured neural pathways that are necessary for proper development of the nervous system (3).

The molecular signals produced by the binding of CAMs trigger signaling cascades within target cells which subsequently activate cellular kinases and raise cellular levels of Ca_2+, both of which influence neuronal migration. Migration of neurons is also influenced by chemical gradients that are established when target cells secrete soluble factors. Secreted proteins, made by the target cell, can either attract or repel a growing axon, thereby guiding the formation of neural connections (3).

Cell Adhesion Molecules (CAMs)

An important group of proteins that are involved in building the correct neural circuitry are the cell adhesion molecules (CAMs). CAMs are integral membrane proteins that facilitate connection of one cell to another through binding of CAMs on one cell to

CAMs on an adjacent cell (4). CAMs are very important during development for guiding axons in finding their intended target neuron (3). There are three major families of cell adhesion molecules having three common features: an extracellular domain, a transmembrane domain, and a cytoplasmic domain. Members of the three families of CAMs are specialized to specific tissues and functions and they include: 1. the cadherins, which bind to other cadherins (i.e. homophilic binding) in a calcium-dependent manner, are vital in early embryonic tissue development, 2. selectins, which are mainly expressed on leukocytes and endothelial cells and are involved in immune reactions, and 3. immunoglobulin-like adhesion molecules, which can undergo both homophilic and heterophilic binding thereby binding a wide diversity of cell types that are involved in early neural development. The integrins, a related class of proteins, have a structure similar to the CAMs, but they are substrate adhesion molecules. Integrins play an important role in cell signaling by providing a link between the cytoskeleton and the extracellular matrix, binding to components of each (4).

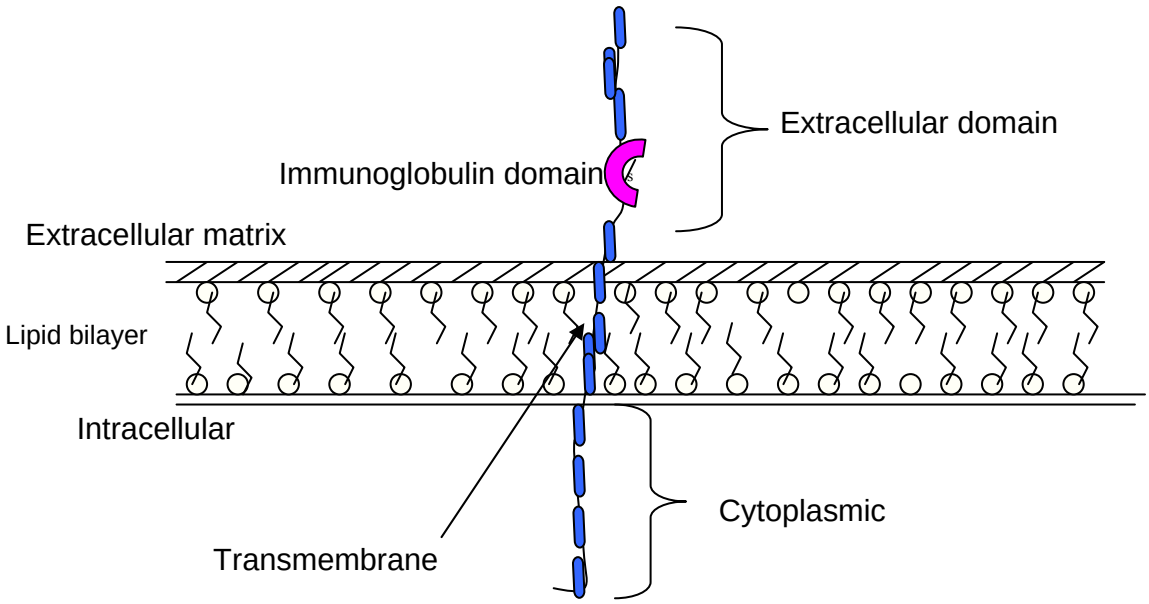


Figure 1. Immunoglobulin Superfamily Protein (Lawson, 2003)

Ig molecules consist of two light and two heavy protein chains, each of which has a constant domain and a conserved domain (6). The immunoglobulin family of CAMs includes a large group of molecules that are generated from a smaller number of genes by alternative splicing; these proteins bind to various cellular receptors in a calcium-independent manner. Immunoglobulin (Ig) CAMs all contain one or more Ig domains, which are typically 70-110 amino acids long with conserved cysteine and tryptophan residues that are involved in binding to a wide variety of different CAMs on adjacent cells (6). The most extensively studied members of this group are the neural cell adhesion molecules (N-CAMs) which are expressed primarily in nervous tissue and are involved in early neural development (4).

Neural cell adhesion molecules (N-CAMs) of the Ig superfamily maintain groups of cells at key sites during early development and in adult organisms (7). The neural cell adhesion molecules are expressed primarily, but not exclusively, in nervous tissue and are

involved in setting up the early circuitry of the nervous system (4). These N-CAMs of the Ig superfamily maintain groups of cells at key sites in the brain and skeletal muscle by mediating adhesion (through cell-to-cell contact), guidance, and differentiation during neuron growth. Additionally, the homophilic binding of N-CAMS to other N-CAMs on target cells leads to signaling events that result in changes in gene expression (6, 7). The role of N-CAMs in neural development has been studied in invertebrate species, such as *C. elegans*, as well as vertebrate species, such as *M. musculus* (6). N-CAMS are Ig-like neuronal surface glycoproteins that bind to other CAMs. In addition to their adhesive properties, binding of N-CAMs can affect intracellular signaling. One example of intercellular signaling involving N-CAMs is a calcium-dependent signal transduction pathway that is triggered by N-CAMs binding in homophilic manner; this pathway ultimately leads to the activation of the fibroblast growth factor receptor (FGFR) (8). The FGFR family of receptor tyrosine kinases is expressed widely in the developing nervous system and activation of the receptor results in neurite outgrowth (8).

Another pathway that involves homophilic N-CAM binding leads to the activation of the mitogen-activated protein kinase pathway. In addition, cyclic adenosine monophosphate and protein kinase A are involved in N-CAM-mediated signaling (8). Also, several downstream effector molecules leading to N-CAM-mediated cellular endpoints have been established, including transcription factors and regulators of the cytoskeleton (8). Their ability to influence developmental events, including cell migration, proliferation, and differentiation can therefore result both from their adhesive as well as their signaling properties. Mutations in N-CAM genes can lead to human genetic diseases including mental retardation and nervous system disorders (7).

Down syndrome cell adhesion molecules (DSCAMs) are a specific type of N-CAM that is vital for early neural differentiation and development in organisms as diverse as *Drosophila* and humans (9). DSCAMs are involved in guiding dendritic and axonal processes during early neural development of the central and peripheral nervous systems in humans (9). An overabundance of these DSCAMs is believed to contribute to the mental retardation and some of the other health problems associated with Down syndrome in humans (9).

Genetic Contributions to the Development of the Nervous System

In order to form precise neural connections, it is necessary for an organism to have many different CAMs in its protein repertoire. However, this poses a problem for organisms that have a relatively small genome size; these organisms cannot have a single gene to code for each specific CAM. A DSCAM homolog in *Drosophila melanogaster* has more than 38,000 different alternative splice products (10), which may solve the problem of having only a few genes with which to create a complex nervous system. *Drosophila* accomplishes this by having its alternatively spliced exons arranged in four clusters, each of which is spliced in a mutually exclusive manner. The other exons in the gene are constitutively spliced in the mRNA (10). On the other hand, the human, with more genes than *Drosophila*, has DSCAMs with only a few splice variants (11); even though humans presumably have a much more complex nervous system than *Drosophila.*

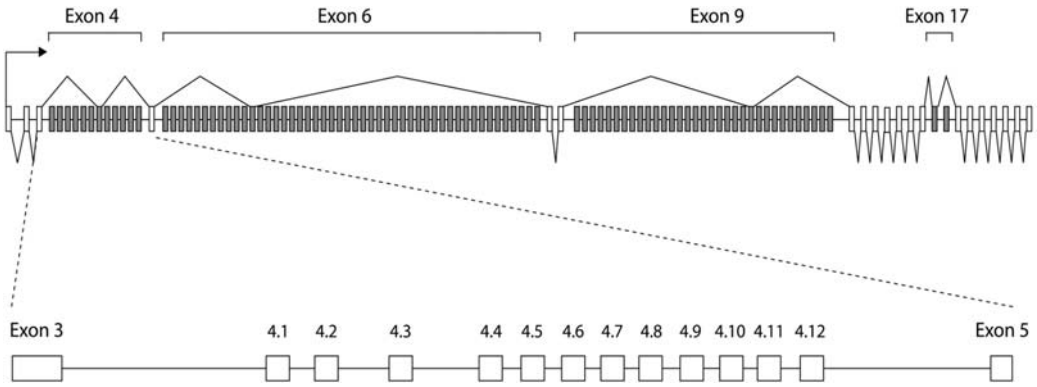


Figure 2. Organization of the *D. melanogaster Dscam* gene (Celotto *et al*., 2001)

Innate Immunity in *C. elegans*

Unlike vertebrate species that have both an innate immune system and a highly developed acquired immune system involving the formation of antibodies, *C. elegans* has only an innate immune system for immune protection against invading pathogens. Antibodies that are responsible for an acquired immune response are members of the immunoglobulin superfamily of proteins. This type of response is highly specific; each antibody only recognizes a single antigen. In higher vertebrates, there is a highly diverse protein repertoire of antigen-specific receptors that are created by somatic gene rearrangement and clonal selection. Invertebrates, however, do not make the large array of diverse antibodies seen in vertebrates. Instead, they may use the method of alternative splicing of an Ig-like molecule to produce a large protein repertoire to deal with specific immunity (12).

Because *C. elegans* lacks any cellular immunity, these animals produce peptides called antibacterial factors (ABFs) that act in an antimicrobial capacity and are constitutively expressed in the animal. Regulation of gene expression is altered in *C. elegans* following infection by a pathogenic microorganism. Bacteria that infect the

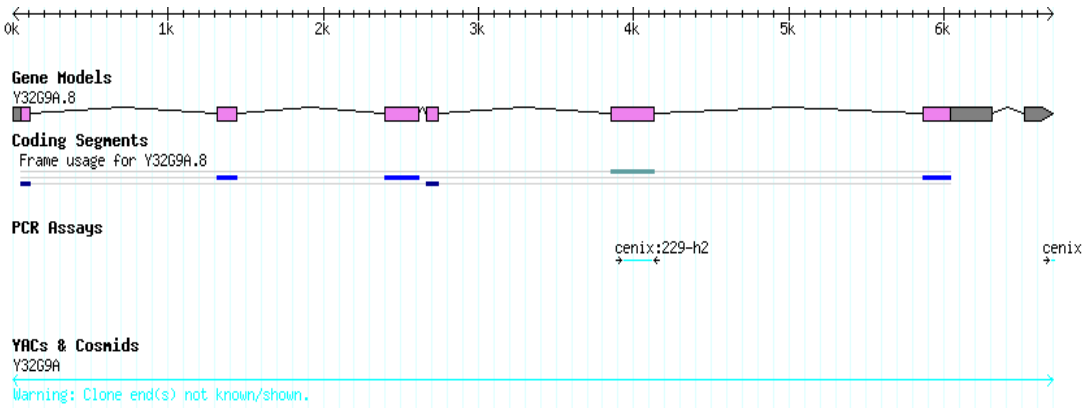
worm's intestine induce genes that code for antimicrobial peptides such as lysozymes. Cells of the intestinal wall are thought to secrete these antimicrobial peptides into the intestinal lumen so the peptides can specifically target any invading pathogens that are present there (13).

The largest group of surface receptor proteins found in animals is the immunoglobulin (Ig) superfamily of proteins. These proteins may play several roles including cell-adhesion and cell signaling (12). In *Drosophila* a Down syndrome cell adhesion molecule (DSCAM) gene, a member of the Ig superfamily, has been identified as an important component responsible for wiring neural circuitry (12). This gene undergoes alternative splicing to produce more than 19,000 protein variants differing in their extracellular domains; this would allow for recognition of numerous different ligands on diverse surface receptors. Watson et al. found that DSCAM is also expressed in *Drosophila* fat body cells, cells which constitute an important component of the fly's immune system (12). Fat body cells are also important in humoral immunity in *Drosophila* because they secrete antimicrobial proteins that provide some defense against pathogens. Microarray analysis of gene expression revealed that different and distinct subsets of exon sequences were found in fat body cells compared to cells derived from brain tissue (12). Several secreted forms of the DSCAM protein were also found in extracts from fat body cells and are thought to circulate in the hemolymph (12)

C. elegans has several different cell-signaling pathways that are important to the worm's innate immune system. One such pathway is the DBL-1 pathway, a pathway that is homologous to the TGF-β pathway in mammals; animals that have mutations in this pathway are vulnerable to infection by pathogenic bacteria. Another important pathway,

the DAF-2 (abnormal Dauer formation)/IGF (insulin-like growth factor) pathway, regulates aging in *C. elegans*. DAF-16, a transcription factor, is inhibited by DAF-2. Several antimicrobial genes are the targets of DAF-16, so inhibition of this transcription factor would presumably downregulate these important genes (14).

Toll-like receptors (TLRs) are an important component of the innate immune system of many organisms. These proteins are transmembrane proteins that have an extracellular leucine rich repeat domain and an intracellular Toll/interleukin-1 receptor (TIR) domain (15). These TIR-containing proteins activate signal transduction pathways that are important for innate immunity in animals as well as plants (16). C. *elegans* has one Toll homolog, *tol-1,* and homologs to other NFkB signaling genes that are involved in the Toll signaling pathway. Pujol *et al* (2001) found that *tol-1* functions in pathogen recognition and avoidance in *C. elegans*.



***www.wormbase.org**

Figure 3. Organization of Exons in the Y32G9A.8 Gene in *C. elegans*

Characterization of Y32G9A.8

Y32G9A.8, a putative expressed gene in *C. elegans*, was identified by performing a BLAST search using a known human IgG domain from a Herpes Simplex 1 receptor (17). The Y32G9A.8 gene is located on chromosome V, and the genomic DNA contains 6,042 nucleotides, with 6 predicted exons. Y32G9A.8 is related to the zig gene family that is involved in axonal development in *C. elegans*. Surprisingly, Y32G9A.8 is more closely related to a DSCAM in humans than any *C. elegans* gene (17). Figure 3 shows the organization of Y32G9A.8 in *C. elegans*.

* www.ncbi.nlm.nih.gov

Figure 4. Organization of CHD2, a Human DSCAM with Two Isoforms

The predicted protein of Y32G9A.8 has 304 amino acids and has 41.1 % similarity to the amino acid sequence of CHD2-52 (a DSCAM in humans) in an IgCAM domain consisting of 56 amino acids. CHD2-52 is a transcript variant of CHD2, a DSCAM precursor; this variant lacks an alternate segment compared to variant 1 that causes a frameshift mutation. The resulting isoform (CHD2-52) is shorter and has a distinct C-terminus compared to isoform CHD2-42. Isoform CHD2-52 may be secreted rather than membrane-bound as is isoform CHD2-42 (17). The organization of these human DSCAM isoforms and their coding segments is illustrated in Figure 4. ZIG-1, a *C. elegans* protein containing an IgCAM domain, has 28.2 % similarity to Y32G9A.8

(17). The predicted protein of Y32G9A.8 has three Ig domains and one Ig-like domain, but the protein has not been isolated (17). Since the size of the *C. elegans* genome is similar to that of *Drosophila,* one might predict that Y32G9A.8 gives rise to many alternative splice variants. But since this putative gene is more similar to the human DSCAM, it may only have a few variants. Figure 5 shows the predicted protein with its functional domains.

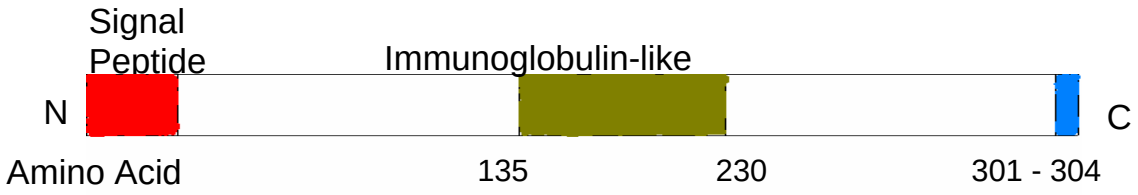


Figure 5. TheY32G9A.8 Putative Protein in *C. elegans*

First, it was necessary to find out if Y32G9A.8 is a gene that is actually expressed in *C. elegans*. Previous work in the lab partially characterized the expression of this gene. Northern dot blot and RT-PCR (using a probe and primer for exon 4) analysis showed that Y32G9A.8 is transcribed at all larval stages in *C. elegans* (17). After determining that Y32G9A.8 is a transcribed gene, it was important to determine if it is necessary for survival and normal function in *C. elegans*. RNAi experiments, using both soaking and microinjection, indicate that the gene product is essential for normal function in the adult worm (17). Treated worms exhibited a high mortality rate, while those that survived exhibited uncoordinated, lethargic movement. Control animals, alternatively, exhibited a considerably higher survival rate and normal phenotype (17). I have attempted to extend this work and determine the expression pattern of Y32G9A.8 by

isolating the promoter and characterizing it. I have created a GFP reporter construct by fusing the gene's putative promoter to a GFP coding region from a GFP expression vector. By determining the expression pattern of this gene, we will be one step closer to determining this gene's normal function in *C. elegans*. I initially expected to see Y32G9A.8 expressed at high levels in cells of the nervous system, and I expected to find that multiple splice variants were produced.

Y32G9A.8, a partially characterized gene in *C. elegans*, was further characterized by determining the gene's expression pattern and the number of splice variants produced. Previous bioinformatics analysis and RNAi experiments indicate that this gene is a DSCAM homolog that is important for normal development in *C. elegans*; these analyses suggest that the gene is expressed in the nervous system. Results of the expression pattern analysis, however, indicate that Y32G9A.8 is expressed in the worm's intestine. Because expression seems to be localized to the worm's intestine, it is likely that this gene is involved in innate immunity. Proteins involved in *C. elegans'* innate immune system are normally expressed in the animal's gut. Furthermore, analysis of the number of splice variants indicates that the gene is structurally more similar to human DSCAMs than those of *Drosophila*.

MATERIALS AND METHODS

One strain of animal was used for DNA isolation, RNA isolation, and microinjection. *Him-5* animals exhibit wild-type phenotype, but they produce 20% males in their offspring due to non-disjunction of the X chromosome. Typically, wild-type animals produce 0.1% males. The basic C. elegans anatomy is shown in figure 6.

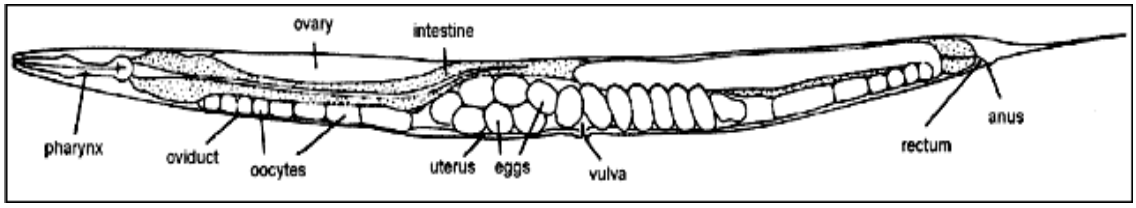


Figure 6. Anatomy of a Hermaphrodite (avery.rutgers.edu)

him-5 worms were grown on NGM agar plates (0.3% NaCl, 2.5% peptone, 1.7% agarose, 5µg/mL cholesterol, 1mM CaCl, 1mM $MgSO_4$, 25mM $KHPO_4$) containing OP50 bacteria (an *E. coli* strain) as food. Worms were transferred to fresh NGM plates every 7-10 days to prevent overcrowding of the plates. Worms were kept in a sterile 20˚C incubator.

Genomic DNA Isolation

Genomic DNA was isolated from a mixed population of *him-5* worms. Five mL of 200 mM Tris-Cl/100 mM EDTA/400 mM NaCl was pipetted onto four 60x15 mm NGM plates containing *him-5* worms. The solution was pipetted and put into two 15-mL sterile Falcon tubes. The worm solution was centrifuged in an IEC clinical centrifuge at a setting of 5 for 3 minutes. The supernatant was removed, and the worm pellet was washed by the addition of 5 mL of 200 mM Tris-Cl/100 mM EDTA/400 mM NaCl to the pellet. The solution was pipetted up and down several times and re-centrifuged as before, removing the supernatant after centrifugation. This wash was repeated 3 more times to remove any remaining bacteria. The worms were then transferred to a 1.5-mL microcentrifuge tube and centrifuged at 12,000 g for 1 minute at room temperature. The liquid was then removed from the pellet by aspiration. The pellet was placed on dry ice for 10 minutes and then thawed by the addition of 0.5 mL nematode lysis buffer

equilibrated to 65˚C. The mixture was pipetted up and down through a cut-off P-1000 pipette tip and then inverted to mix. After mixing, the nematode mixture was incubated at 65˚C for 1 hour and inverted every 20 minutes. The worm lysate was extracted twice with one volume TE-saturated phenol. The lysate was then extracted with one volume of chloroform to remove any remaining phenol. Worm DNA was precipitated with 1/3 volume 7.5 M ammonium acetate (pH 7.4) and 2.5 volumes absolute ethanol. The DNA was incubated at -20˚C for 30 minutes and then centrifuged at 14,000 rpm for 15 minutes. The DNA pellet was washed with 70% ethanol and re-centrifuged for 10 minutes, after which the pellet was allowed to air dry for 5 minutes. Fifty µL of TE buffer was added to the pellet to resuspend the DNA. The DNA solution was diluted 1:1000 and the concentration was measured using a UV spectrophotometer.

GFP Reporter Constructs and Expression Analysis

Reporter gene constructs are often used to produce transgenic animals for analysis of gene expression patterns. GFP, the green fluorescent protein isolated from the jellyfish *Aequorea victoria,* is used to visualize gene expression patterns in a variety of animal species including *C. elegans* (18). In a reporter gene construct, the promoter of a gene of interest is fused to a reporter gene, such as GFP, whose gene expression is clearly visible. In the transgenic animal, the gene of interest should be present wherever the reporter gene is expressed. The sensitivity of GFP reporter constructs allows one to visualize gene expression in a single cell (19), and because worms are semi-transparent, visualization of GFP expression is straightforward.

There are two types of reporter gene constructs: ones that show transcriptional expression and those that show translational expression. Transcriptional reporter gene

constructs consist of a gene's promoter fused directly to the reporter gene, whereas translational reporter constructs consist of the promoter, the entire gene, and the reporter gene fused together (20). Traditional reporter gene constructs are produced by subcloning a PCR product (promoter or promoter + gene) into a GFP expression plasmid. This subcloning strategy, however, is time-consuming and not feasible for high-throughput gene expression analysis.

An alternative method for creating GFP reporter constructs is that of fusion-PCR, or stitching. Fusion-PCR is a quick, effective method for producing GFP reporter constructs on a large scale (19). The PCR fusion method negates the need for timely subcloning and DNA purification methods. With fusion PCR, two separate PCRs are performed in parallel; one reaction amplifies the gene-of-interest's promoter, while the other amplifies GFP from a GFP expression vector. The concentrations of each of these products are estimated by gel electrophoresis, and approximately 10-50 ng of each PCR product is used as the template for the fusion PCR. Nested primers are used for the fusion PCR. The downstream primer for the gene's promoter, primer B, is designed with a 24 bp overhang which is complementary to the 5' end of the GFP PCR product. This overlapping region allows the two separate PCR products to hybridize and serve as a template for the subsequent fusion or stitching of the products (18). With this method, a reporter construct can be constructed and ready for microinjection within a single day, providing that the correct PCR conditions are known and the reactions are successful. Figure 7 illustrates the method for creating a reporter construct by PCR fusion.

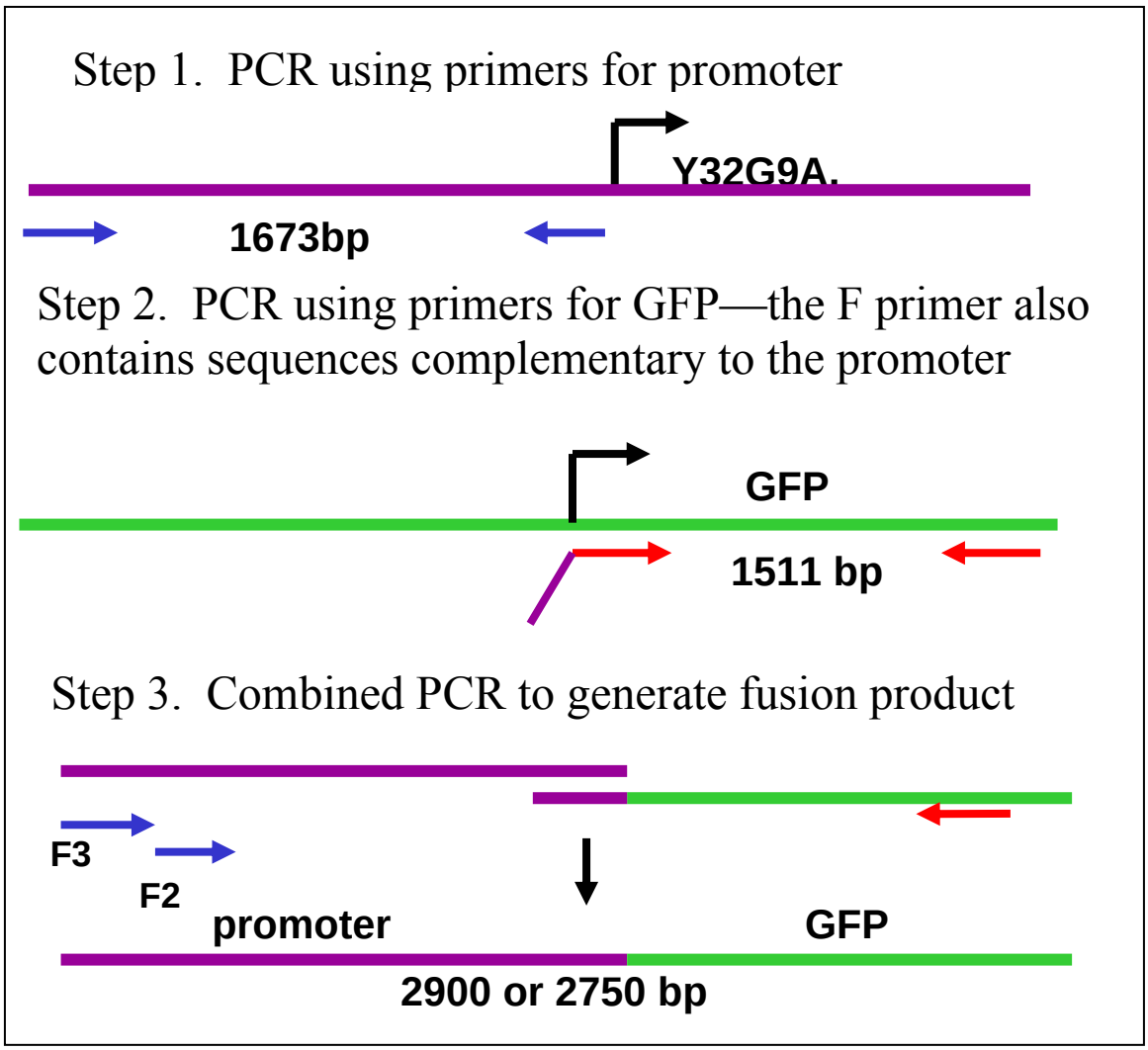


Figure 7. Diagram of Fusion PCR Method. The templates are worm genomic DNA (PCR#1) and the GFP expression vector pPD117.01 (PCR #2). In PCR #3, nested primers were used to create the GFP promoter fusion product using products from PCRs 1 and 2 as the template.

Promoter Isolation and Vector Construction

In order to look for the spatial and temporal gene expression pattern of Y32G9A.8, I generated a GFP fusion vector using a novel method developed by Hobert that utilizes PCR to generate overlapping sequences between the promoter sequence and the GFP coding sequence (18). Both the promoter region and the GFP region were first

amplified separately. Then, since the 3' end of the promoter sequence and the 5' end of the GFP sequence were complementary (resulting from the primers used), I combined the two products and used primers on the 5' end of the promoter and 3' end of the GFP to make a composite DNA sequence that consisted of the promoter for Y32G9A.8 fused to the coding region for GFP. The construction of this fusion product is outlined in figure 6.

PCR was performed on the genomic worm DNA using a set of primers that were named PVLR/05-F1 & PVLR/06-R2. The primers were designed using the Genome Sciences Centre's sequence annotation-directed PCR primer design program (http://elegans.bcgsc.bc.ca/gfp_primers/), and they were designed to amplify the putative promoter of the Y32G9A.8 gene, a region upstream from the gene that is 1685 bp in length. Genomic worm DNA that was previously isolated was used as the template for PCRs using the PVLR primer set. The final concentration of $MgCl_2$ that was used for the reactions was 3 mM. A series of reactions with varying concentrations of $MgCl_2$ were prepared and run in an Eppendorf Mastercycler Gradient thermal cycler. The temperature gradient that was used for the annealing temperature of the reactions was 47° -67° C. Reactions with a final $MgCl_2$ concentration of 3mM and an annealing temperature of 59.1° C produced the most prominent band when samples were run on a gel. For subsequent reactions, the thermal cycler was set for the following cycle: 5 minutes at 95˚C, then 35 cycles of 1 minute at 95˚C, 1 minute at 59.1˚C, and 1.5 minutes at 72˚C. The PCR products were electrophoresed on a 1% agarose gel stained with ethidium bromide.

PCR was also performed using pPD117.01, a GFP expression plasmid, as a template. Figure 8 shows a map of the pPD117.01 expression plasmid. The primers for

this PCR were designed using the Primer3 Input program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and they were called pPD117-F and pPD117-R2. A nested primer, pPD117-R1 was also designed using this program. These primers were designed to amplify a region approximately 1500 bp in length which included the GFP coding region. The forward primer pPD117-F was designed to have a 24-bp region on its 5' end which overlaps with the 3' region of the PVLR promoter fragment. Products from this reaction were electrophoresed on an agarose gel to confirm their size and estimate their concentration.

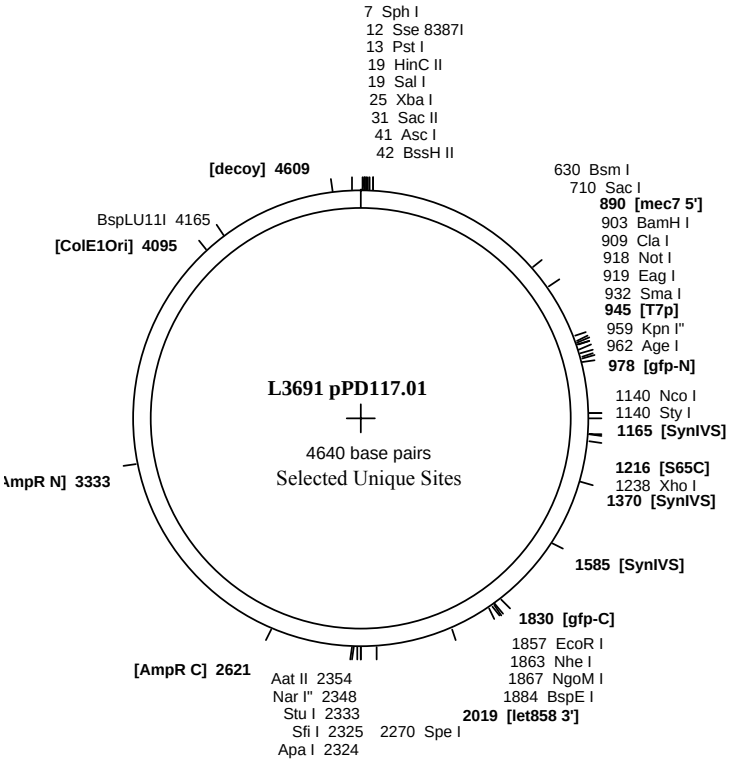


Figure 8. GFP Expression Plasmid pPD117.01. pPD117.01 was used as a template to amplify its' GFP coding region which was used to create a GFP promoter fusion product. The primers used, pPD117-F and pPD117-R2, were designed to amplify a region approximately 1500 bp in length which includes the GFP coding region as well as a 3' UTR (let858) that facilitates GFP expression.

The resulting PCR products from the PVLR and pPD117 reactions were subsequently used as templates for the fusion PCR. One µL of each product was added to the fusion reaction to serve as the template. The nested primers PVLR/05-F2 (or F3) and pPD117-R1 were used in the fusion PCR. These reactions were expected to produce products approximately 2900 or 2750 bp in length, respectively. The fusion PCR method that was used is outlined in Figure 6. The sequences of the PCR primers are given in Table 1.

Table 1. PCR Primers used for Fusion PCR

	Sequence
PVLR/05-F1	5'AGATGAAACCCGACAGGAAAT 3'
PVLR/05-F2	5'ACGAAAAAGCGGTGGAAAAT 3'
PVLR/05-F3	5'TGCGCAAAACGTATTCTCAG 3'
PVLR/06-R2	5'TCTTGATTGGCTCTTCTGTACG 3'
pPD117-F	5'ACCGTACAGAAGAGCCAATCAAGAGGTGCCCTATAGTGAGTCGT3'
pPD117-R1	5'CTCATCGTTCGCCTAATTTC 3'
pPD117-R2	5'TGTCTCATGAGCGGATACAT 3'

* overlapping region

Microinjection and Transformation

Transgenes can be introduced into an organism in order to observe their effects *in vivo*. This type of genetic manipulation has been used in bacteria, yeast, cultured mammalian cells, as well as multicellular organisms such as *Drosophila* and *C. elegans*. In the case of multicellular organisms, the transforming DNA is usually delivered by microinjection into a fertilized egg, or, with *C. elegans*, into the gonad. By introducing transgenes, one can study the temporal and spatial expression of newly introduced sequences (21).

C. elegans is an ideal organism for the use of DNA transformation by microinjection. The injection of both linear and circular DNA into adult hermaphrodites can lead to the formation of stably inherited extrachromasomal DNA arrays and the creation of transgenic animals which can be used for further screening (22). The most effective method of producing transgenic worms is by injecting DNAs into the distal part of the gonad. Because *C. elegans* has a syncytial gonad, injected DNAs can be passed on to many offspring (22).

The GFP-promoter fusion DNA was microinjected into the syncytial gonad of adult animals. 250 ng of reporter DNA was dissolved in 5 μL of 1X microinjection buffer (2% polyethylene glycol, 20mM potassium phosphate, pH 7.5 and 3mM potassium citrate, pH 7.5). The DNA concentration was increased to 100 ng/μL using genomic worm DNA as carrier DNA. The needles used for microinjection were physically broken by pushing the needle against the edge of a coverslip. This was done after loading the needle with the prepared DNA solution. Several adult hermaphrodites were mounted on a 2% agarose pad containing Halocarbon microinjection oil. Once the animals were stuck to the pad, the agarose pad was placed on the injection table and the animals were injected with the DNA solution. Injected animals were recovered to a fresh NGM plate containing food following a brief soaking (~10-15 min.) in recovery buffer (M9 plus 4% glucose). Offspring of the injected animals were observed 48 and 72 hours following microinjections. Animals were observed for GFP expression using a Zeiss fluorescent stereoscopic microscope.

Analysis of Alternative Splice Products

Total RNA was isolated from a mixed population of *him-5* worms growing on NGM plates seeded with OP50 bacteria. Worms were rinsed from ten 100x15 mm NGM agar plates using 5 mL of DEPC-treated water to remove them. The worms were pipetted into 15 mL sterile Falcon tubes. The worm solution was centrifuged at a setting of 5 in an IEC clinical centrifuge for 3 minutes, after which the supernatant was removed and the pellet was washed 4 times with 10 mL DEPC-treated water. The pellet was aspirated to remove as much liquid as possible and placed on dry ice for 10 minutes. The worm pellets were thawed on ice and 4 mL of Trizol was added to 1 mL of packed worms in each 15-mL tube. The tubes were vortexed vigorously for 1.5 minutes, and the worm mixture was frozen and thawed for 2 cycles on dry ice. After the final freezing, the pellets were thawed on ice, and an additional 2 mL Trizol was added to each tube and vortexed vigorously for 1.5 minutes. Two mL chloroform was then added to each tube, and the tubes were shaken for 15 seconds and incubated at room temperature for 3 minutes. The worm lysate was aliquotted into RNase-free tubes and centrifuged at 12,000 g for 15 minutes. The top aqueous layer was removed to fresh tubes, and an equal volume of RNase-free isopropanol was added to each tube and mixed by inverting. The mixture was allowed to incubate at room temperature for 10 minutes, after which the samples were centrifuged at 12,000 g for 10 minutes. Following centrifugation, the RNA pellets were washed with 75% ethanol (made with DEPC-treated water) and re-centrifuged at 7500 g for 5 minutes. After washing the pellets, the supernatant was removed, and the pellets were air-dried for 10 minutes. RNA pellets were dissolved in 10 μL RNase-free water and incubated at 60˚C for 10 minutes. The RNA solutions were combined in one

1.5 mL microcentrifuge tube, and the concentration of the RNA solution was measured using a UV spectrophotometer. A sample of the total RNA solution was electrophoresed on a non-denaturing agarose gel.

RT-PCR was performed using the StrataScript One-Tube RT-PCR kit and protocol. The primers used, Y32G9A.8-F and R, were designed to reverse-transcribe and amplify 910 bp of the 915 bp Y32G9A.8 coding region. Primers Y32G9A.8-F and R were designed to base pair with exons 1 and 6 respectively. Another primer, F2-Y32G9A.8, was designed approximately 50 bp upstream from exon 1 and was expected to amplify approximately 960 bases when used with the Y32G9A.8 reverse primer. Figure 9 illustrates the location of the primers in relation to the gene transcript. A control reaction was prepared by adding the following to a PCR tube on ice: 40.5 µL RNase-free water, 5 µL 10X RT-PCR buffer, 1 µL control primer set (200 ng/µL), 1 µL dNTP mix (40 mM), 1 µL control mRNA (500 bp). The experimental reaction was prepared by adding the following to another PCR tube on ice: 39.5 µL RNase-free water, 5 µL 10 PCR buffer, 1 µL F-Y32G9A.8 primer (60 µM), 1 µL R-Y32G9A.8 primer (60 µM), 1 µL dNTP mix (40 mM), 3 µL total worm RNA (0.26 µg/µL). The StrataScript RT enzyme was diluted by adding the following to an RNase-free microcentrifuge tube on ice: 6.2 µL RNase-free water, 0.8 µL 10X RT-PCR buffer, 1 µL StrataScript RT (20 U/µL). One µL of the diluted RT enzyme was added to each PCR tube, followed by the addition of 0.5 µL Easy-A HiFi PCR cloning enzyme. The samples were gently mixed, avoiding bubbles. The samples were placed in a thermal cycler that was set as follows: 42˚C for 60 minutes (RT reaction), 95˚C for 1 minute, followed by 40 cycles of 95˚C for

30 seconds, 53.5˚C for 30 seconds, and 68˚C for 2 minutes. The RT-PCR samples were electrophoresed on a 1% agarose gel stained with ethidium bromide.

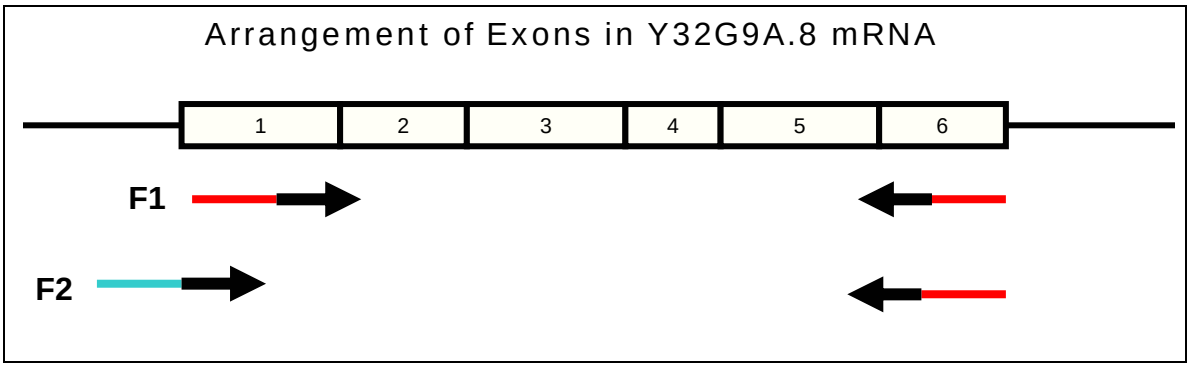


Figure 9. Location of primers for RT-PCR within Y32G9A.8 transcript. Arrows indicate the location of primers used for RT-PCR. Primer Y32G9A.8-F is located 5 bp downstream from the transcription start site in exon 1. Primer Y32G9A.8-F2 is 50 bp upstream from the transcription start site. The reverse primer, Y32G9A.8-R (not labeled) is located at the end of exon 6 and was used for both RT-PCR reactions. Total RNA was used as the template.

RESULTS

Construction of the GFP fusion proved to be difficult. Throughout the experiment, the amplification of the promoter sequence was challenging and involved a lot of trial and error using different conditions of $MgCl_2$ concentration and pH. However, this obstacle was overcome and sequencing of the PCR fusion product confirmed that I had indeed inserted the promoter region of Y32G9A.8 in frame with the GFP coding region (data not shown).

Constructing the Promoter: GFP Fusion Product

The primers used to amplify the promoter region are located at -1 bp (PVLR/06-R2) and -1673 bp (PVLR./05-F1) from the translation start site, so the predicted size of the PCR product is 1673 bp. A product of approximately 1700 bp in length was

generated following PCR of the Y32G9A.8 promoter (Fig. 10) that corresponds with the predicted size. Similarly, for the GFP PCR, the predicted size is 1511 bp, and following PCR using the PPD117-R2 and F primers a product of approximately 1500 bp in length, was detected (Fig. 10).

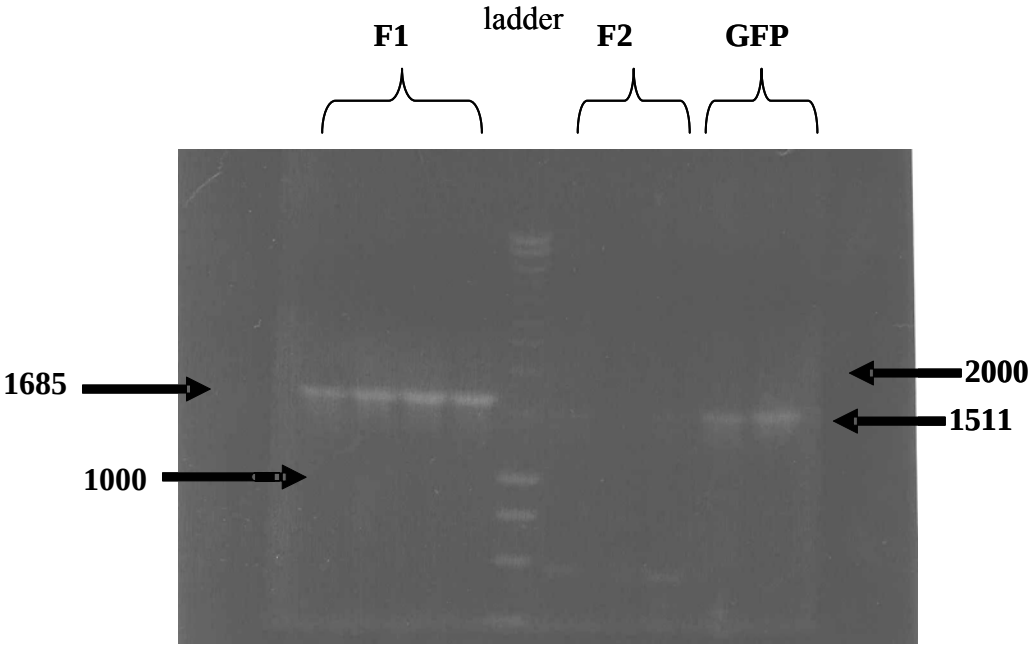


Figure 10. PCR to amplify Y32G9A.8 promoter sequence and GFP coding region. Lane 5 was loaded with 5 µL of Bioline's Hyperladder DNA marker. Lanes 1-4 contain the Y32G9A.8 promoter amplified by PCR using PVLR/05-F1 as the forward primer. Lanes 6-8 contain the Y32G9A.8 promoter amplified using PVLR/05-F2 as the forward primer. Lanes 9 and 10 contain the GFP coding region amplified using the PPD117 primer set.

Following PCR of the promoter, the amplified product was separated by gel electrophoresis and purified from an agarose gel. The purified PCR product was sent to the Biotechnology & Drug Design Core Facility at Georgia State University for DNA sequencing. This was done to ensure that the sequence of the amplified promoter matched the gene's published sequence which is available at www.wormbase.org.

Sequencing of the promoter PCR product confirmed that the promoter matched the published sequence (results not shown).

The next step was to generate the fusion product by combining the two individual pieces of DNA and performing the next round of PCR. The fusion, or stitching, PCR resulted in a product approximately 2900 bp in length for the reaction using PVLR/05-F2 as the nested primer (Fig 11). When PVLR/05-F3 was used as the nested primer, the reactions produced products of the expected size (~2750 bp).

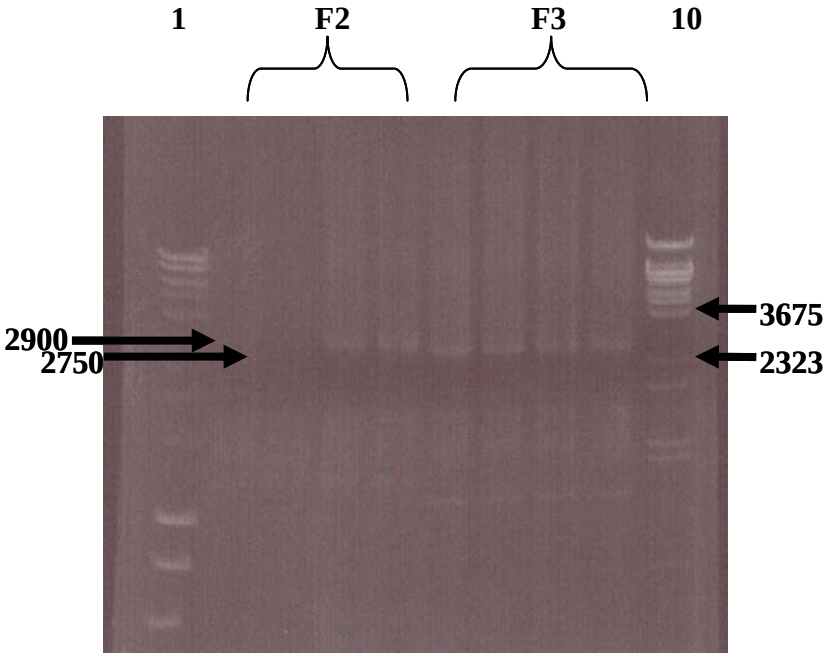


Figure 11. Fusion PCR to create Y32G9A.8 promoter:GFP fusion product. Lane 1 was loaded with 5 µL of Bioline's Hyperladder DNA marker. Lanes 2-5 contain the fusion PCR product using PVLR/05-F2 as the nested primer. Lanes 6-9 contain a fusion PCR product using PVLR/05-F3 as the nested primer. Lane 10 contains BstE II marker.

Gel electrophoresis results indicate that a PCR product of the correct size was produced with each PCR reaction. Both the PVLR and GFP PCRs produced products of the expected size. When the two PCR products were used as templates for the fusion

PCR, it appears that the appropriate fusion product was produced based on the size of the fragments (2900 bp for the F2 forward primer and 2750 bp for the F3 forward primer).

Observation of Fluorescence

Once the promoter fusion product was successfully produced, it was injected into the syncytial gonad of *C. elegans* in order to observe the gene's expression pattern in offspring of the injected animals. Adult worms were microinjected with a solution containing the GFP fusion DNA in 1X microinjection buffer. After 48 hours, the worms were observed under a Zeiss fluorescent dissecting microscope for the presence of fluorescence in the form of GFP. Twenty-four adult hermaphrodite animals were injected with the promoter fusion DNA, 14 of which survived 24 hours following microinjection. Over 150 offspring were observed, only one of which showed the following GFP expression pattern. This was a larval animal, pictured in figure 12, which showed GFP expression in the anterior region of its intestine. When the reporter-fusion construct was microinjected into adult animals, GFP expression was observed in the progeny; this indicates that the PCR fusion was successful. Because GFP expression was observed, this also suggests that the minimal promoter for Y32G9A.8 was included in the reporter construct. This GFP expression pattern was seen on the right side of the animal; however the left side of the animal showed no GFP expression in the same area of the gut, as shown in figure 13. After photographing this animal, it was recovered to a fresh NGM plate with OP50, but it did not survive to produce any offspring.

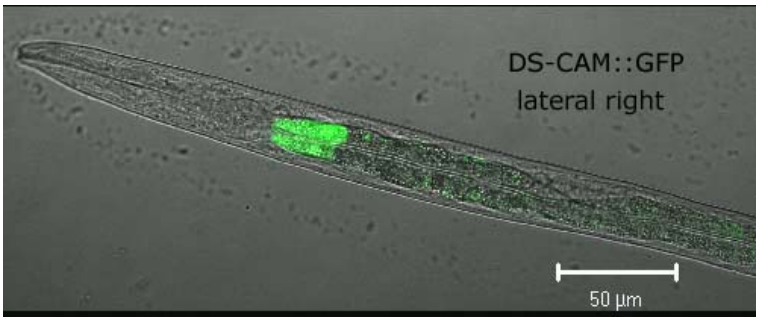


Figure 12. Lateral right view of expression of Y32G9A.8 in *C. elegans* as visualized by microinjection of GFP promoter fusion product (offspring of injected animal is pictured)

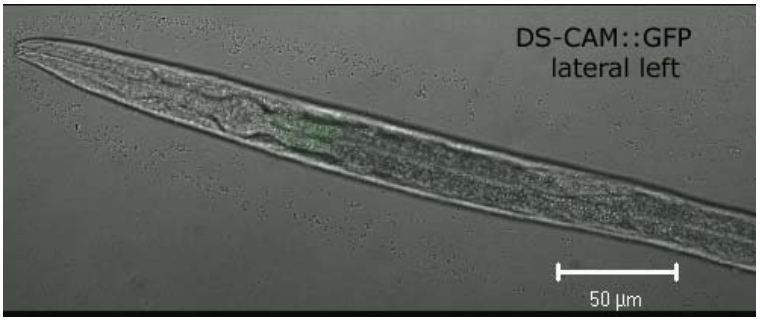


Figure 13. Lateral left view of expression of Y32G9A.8 in *C. elegans* as visualized by microinjection of GFP promoter fusion product (offspring of injected animal is pictured).

Analysis of Alternative Splice Products

Another important question was whether Y32G9A.8 produces a single transcript (similar to the human DSCAM) or multiple transcripts (as in *Drosophila*). To answer this question RT-PCR was performed on total RNA isolated from *C. elegans* and amplified using primers designed for the predicted Y32G9A.8 transcript. Total RNA was electrophoresed on a non-denaturing agarose gel stained with ethidium bromide. The resulting gel, illustrated in figure 14, shows three prominent bands believed to correspond to rRNA bands. The concentration of total RNA was calculated to be 2.6 µg/µL. This isolation of total RNA was used for subsequent RT-PCR experiments.

Figure 14. Isolation of total RNA. Total RNA was isolated using Trizol and electrophoresed on a non-denaturing agarose gel.

RT-PCR primers (Y32G9A.8-F1 & R) were designed to span the length of the Y32G9A.8 transcript. Another forward primer, Y32G9A.8-F2, was designed to be 50 bp upstream from Y32G9A.8-F1 and is located just upstream of exon 1; this was done to determine if the first exon is alternatively spliced. The location of the RT-PCR primers is outlined in figure 9. Total RNA was used as the template for RT-PCR using the StrataScript One-Tube RT-PCR kit and protocol. The positive control reaction included a 500 bp mRNA molecule which was included in the kit. A negative control was performed by omitting the RNA template from the reaction. RT-PCR using the Y32G9A.8-F1 & R primer set produced a single PCR product approximately 910 bp in length, as shown in figure 15. The control reaction produced a 500 bp PCR product as expected. RT-PCR using Y32G9A.8-F2 as the forward primer produced a single product of approximately 960 bp which corresponds to the expected size and is illustrated in figure 15. RT-PCR was repeated using the Stratascript RT-PCR kit, and a single RT-PCR product was produced again (results not shown).

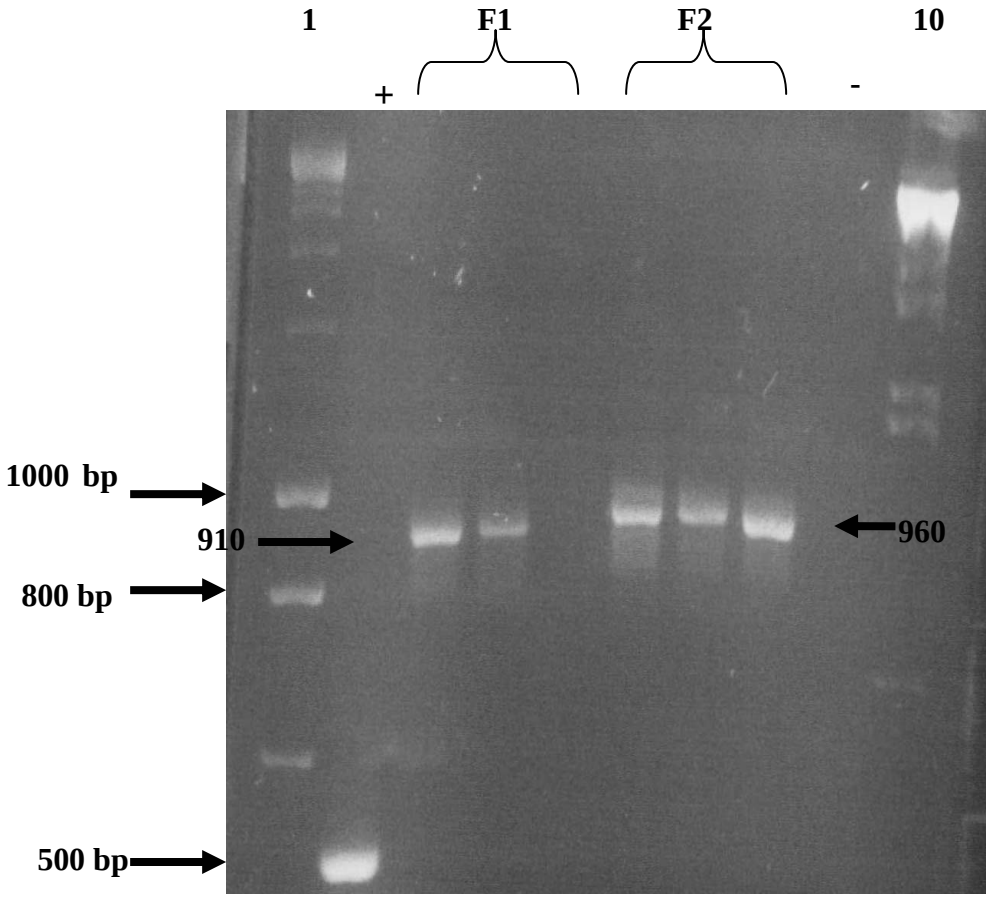


Figure 15. RT-PCR to analyze alternative splicing in Y32G9A.8. Lane 1 contains Bioline's Hyperladder DNA marker. Lane 2 contains the positive control, a 500 bp mRNA. Lanes 3-5 contain RT-PCR using the F1 primer as the forward primer. Lanes 6-8 contain RT-PCR using the F2 primer. Lane 9 is a negative control to which no RNA was added. Lane 10 contains Hind III DNA marker.

DISCUSSION

Earlier work in the lab showed that a novel gene, Y32G9A.8, was expressed in *C. elegans* and that its function was critical for survival (17). RT-PCR and Northern Dot Blot analysis revealed that the gene is expressed in *C. elegans* at all developmental stages. RNAi experiments suggest that the gene is crucial for normal function in *C. elegans*; animals in the experimental group that survived were lethargic and showed delayed response to touch stimuli, and some animals were unable to lay eggs (17). I was interested in extending this knowledge by more precisely determining the location of the expression of this gene in *C. elegans*. To do this, I created a GFP-promoter fusion, microinjected it into worms and observed their offspring for fluorescence at various points in their development. By determining the pattern of expression, I hoped to gain a better idea as to the function of this gene. I also analyzed the alternative transcripts of the gene by RT-PCR to help understand the role of the Y32G9A.8 protein in vivo.

GFP Expression

Before injecting animals with the GFP reporter construct, I predicted that Y32G9A.8 would be expressed in the nervous system. I based my prediction on previous bioinformatics analysis of the genetic sequence. Expression of the gene was localized to the animal's intestine; this unexpected expression pattern leads me to believe that this gene plays a role in the innate immune system of *C. elegans*. Because only a small segment of the putative promoter was included in the reporter construct (~1400 bp), it is possible that including a larger promoter segment would result in a change in the expression of GFP. It may be that the minimal promoter for Y32G9A.8 controls the gene's expression in the gut, while neural expression requires more promoter elements

for expression to occur. Another possibility is that this gene is not expressed in neurons at all despite its homology to other neurally expressed DSCAMs.

In the future, it may be useful to create a reporter construct using a larger segment of the gene's upstream sequence to observe any changes in the gene's expression pattern. This may be done by designing a PCR primer that lies upstream from the forward PVLR primer that was used to create the reporter construct. It would also be very useful to clone the reporter-fusion construct into a vector so that subsequent researchers will have the construct readily available for future experiments.

Alternative Splicing

Many organisms utilize the method of alternative splicing to produce a proteome that is much larger than their genome. This is often necessary in organisms with small genomes in order to create complex systems in the body. Organismal complexity is not directly correlated to genome size. For instance, the human genome is thought to possess about 25,000 genes, only about 33% more than the number of genes in *C. elegans*, although *C. elegans* has much fewer cells (959 somatic cells) than humans. In order to account for the complexity of humans at the genetic level, one must look at the size of the human proteome that is produced from these 25,000 genes, not just the number of genes. The expansion of the human proteome can be achieved by alternatively splicing pre-mRNA, thereby allowing a single mRNA to produce more than one protein (23). The resulting proteins are similar but not identical to each other in structure and function and could, therefore, provide a wide diversity of proteins needed to ensure proper neural arrangement. Organisms such as *Drosophila* use alternative splicing to produce a large

repertoire of proteins from only a few genes. *C. elegans* has also been shown to utilize alternative splicing (23).

Some gene splicing occurs in response to hormonal or developmental cues; this acts as another level of regulation of gene expression (23). There are several methods for producing alternative splice products; these methods include using alternative 5' splice sites, using alternative 3' splice sites, and the use of mutually exclusive exons or retained introns (23). Changes in a protein's primary sequence often result from alternative splicing events; these changes may be slight or dramatic. Another result of alternative splicing is the production of peptide sequences that are prematurely truncated due to the insertion of termination codons in frame (usually due to the insertion of cassette exons) (23). There are several methods that an organism may employ to increase its protein repertoire, thus contributing to the solution of having a relatively small genome (23).

RT-PCR: Analysis of Alternative Splicing in Y32G9A.8

Total RNA was isolated from a mixed population of *him-5* worms using Trizol; this RNA was then used as a template for RT-PCR using primers specific for the predicted coding region of Y32G9A.8. The primers were designed to span the entire Y32G9A.8 transcript; this was done to determine if there are any alternative splice products of this gene. Because only a single PCR product was produced following RT-PCR, it is probable that only a single transcript is produced by the Y32G9A.8 gene. Before performing RT-PCR, total RNA was electrophoresed on a non-denaturing agarose gel to determine if the RNA isolation was successful. The three prominent bands that were present on the gel were believed to correspond to rRNAs. Because the RNA isolation appeared to be successful, the RNA was used in subsequent experiments. The

primer Y32G9A.8-F2 was designed upstream from exon 1, and used with the reverse primer Y32G9A.8-R, to ensure that exon 1 is not an alternatively spliced exon. Because this primer set produced only one RT-PCR product that was about 960 bp, it is likely that exon 1 is not alternatively spliced. Possible future directions include analyzing the exon-intron boundaries in the Y32G9A.8 gene to determine if they follow typical splicing patterns. Because this gene was shown to have only one transcript, it is likely that this gene behaves more like a human DSCAM than a *Drosophila* DSCAM which undergoes extensive alternative splicing due to the organization of many groups of mutually exclusive exons (10).

Possible Function of *C. elegans* DSCAM

It is possible that the *C. elegans* DSCAM homolog, like that of *Drosophila*, has developed a dual function, with function in the immune system as well as in neural development and axon guidance. Known antimicrobial genes that are induced by pathogenic infection, such as several lysozyme genes, have been shown to be expressed mainly in intestinal cells (24). Because analysis of gene expression suggested that the gene is expressed in the worm's intestine, it is likely that Y32G9A.8 is serving some immune function. The study by Watson *et al.* (2005) provides support for this scheme of one molecule acting in two different systems. *Drosophila*, like *C. elegans*, is an invertebrate species that lacks an acquired immune system and thus cannot produce antibodies to facilitate an immune response when pathogens are present. It seems, however, that *Drosophila* has found a way to circumvent this problem by utilizing alternative splicing to create a large protein repertoire and by adapting a new function for the DSCAM gene (12). Watson *et al.* (2005) discovered this possible immune function

of DSCAMs in *Drosophila* by performing immunoprecipitations on fat body extracts; they found a soluble form of DSCAM in the fat body extracts. Fat body cells are an important component of *Drosophila*'s immune system, and the presence of DSCAMs in this tissue indicates that these molecules are performing an immune function in addition to their neural function, indicating a dual function for the DSCAM gene (12).

Due to its location in *C. elegans,* it is likely that Y32G9A.8 has some immune-related function in the worm. *Tol-1*, the toll homolog in *C. elegans*, is required for normal development in *C. elegans*, and it appears to act in a sensory function, allowing the worm to avoid pathogens. Its' expression was seen in mechanosensory neurons, thus supporting the theory that *tol-1* has a sensory role in pathogen avoidance in *C. elegans* (16). If an animal is unable to detect chemical signals secreted by pathogens, the animal is much more likely to become infected with a pathogenic organism.

Another toll-related gene, *tir -1*, was identified due to its similarity to *tol-1* in a conserved intracellular signaling domain, however *tir-1* lacks any transmembrane or receptor domains (25). Results from RNAi studies with *tir-1* showed that knocking down the gene's function caused several ABF genes to be down-regulated. RNAi-treated animals were also more susceptible to fungal and bacterial infection than wild-type animals (25). There is evidence that *C. elegans* uses at least some components of a conserved signaling pathway (the toll pathway) to deal with innate immunity, and it is likely that there are other unrelated molecules (such as DSCAM) that play a similar role in the innate immune system.

Because bioinformatics analysis of Y32G9A.8 suggests that this gene is an N-CAM, which is presumably expressed in the nervous system, it is possible that this

DSCAM homolog is involved in the innervation of the gut. This could explain the high mortality rate seen in the offspring of RNAi treated animals (17). If this gene is involved in the innervation of the gut, knocking out the gene's function with RNAi would cause the digestive tract to become blocked, thus resulting in the animal's accelerated death. Gene expression analysis showed that, using the GFP reporter that I constructed, expression was localized to the animal's gut. It is possible that a GFP reporter containing a larger upstream sequence would result in GFP expression in neurons surrounding the gut as well as the epithelial cells lining the gut.

Conclusions

Results indicate that the genomic organization and structure of the Y32G9A.8 gene is more similar to mammalian DSCAMs than *Drosophila* DSCAMs. Mammals usually produce only one or two DSCAM transcripts while *Drosophila* can have thousands of DSCAM transcripts. RT-PCR analysis revealed that the *C. elegans* DSCAM homolog produces only one transcript; thus, alternative splicing is not taking place. Further analysis, both by molecular methods and bioinformatics, will be necessary to more accurately define the nature of the Y32G9A.8 gene in *C. elegans.*

Results of gene expression analysis indicate that the DSCAM in *C. elegans* may play a role similar to the DSCAM homolog in *Drosophila*. In *Drosophila*, studies show that DSCAMs are expressed not only in nervous tissue, but also in immune cells circulating in the fly's hemolymph. Both invertebrates may have developed a method for tackling the problem of immunity by taking advantage of genes that already serve another, unrelated function in the animal.

Future Directions

In the future, one could create a larger reporter construct in order to see if the gene's expression pattern changes. The method of PCR fusion could be used to create such a reporter construct quickly, and perhaps create a series of reporters. This could be very helpful in more fully understanding the gene's expression pattern *in vivo*. To test the immune function hypothesis, one could infect transgenic animals, expressing a promoter driven GFP reporter, with pathogenic bacteria or fungi and look for up-regulation of GFP expression; such up-regulation of the GFP reporter would indicate that the gene is indeed involved in the worm's innate immune system.

Another method for analyzing gene expression is *in situ* hybridization. This method is time consuming and labor intensive, and it would involve creating a labeled probe to view the gene's expression pattern. Several labeling methods are available for *in situ* hybridization including: autoradiography, fluorescence microscopy, or immunohistochemistry. The latter two methods may be preferred if one wants to avoid exposure to radioactivity. *In situ* hybridization, like microinjection, would allow one to view expression in a whole animal, but animals must be killed and fixed prior to *in situ* hybridization. Because microinjection has proven to be tricky and unreliable, *in situ* hybridization may be a more reliable method for mapping the gene's expression pattern; this may also help to confirm the expression pattern that was observed using the microinjection technique. Another important direction to follow is to isolate the Y32G9A.8 protein. If the protein can be isolated, one may perform a yeast two-hybrid screen to look for other proteins that interact with it. Bioinformatics may prove useful in predicting protein-protein interactions; a protein microarray could also be used to analyze

proteins that interact with the protein of interest. If one knows what types of proteins interact with the Y32G9A.8 protein, one can learn more about how this gene works. Also, isolating the protein could aid in discovering the cellular location of the protein and possibly finding out what, if any, signaling pathways involve this protein.

If a Y32G9A.8 knockout was available, this would be very useful in more fully understanding the gene's function. RNAi experiments suggest that the gene is crucial for normal development and function in the adult animal, but further experiments would help confirm these results and more fully elucidate the gene's function. A gene knockout has been requested from the *C. elegans* Gene Knockout Consortium, but is not yet available. Once available, a gene knockout could prove very important in more fully characterizing this gene. If a Y32G9A.8 knockout animal had an increased susceptibility to infection by pathogens, this would help support the hypothesis that this gene has a role in the worm's immune system.

Determining subcellular localization is important for understanding protein function. Several computational tools for determining the subcellular localization of a protein are publicly available some of which are: Target P (**http://www.cbs.dtu.dk/services/TargetP/**) , which predicts N-terminal sorting signals, and PSORT II (**http://www.psort.org**), which predicts protein sorting signals and localization sites. Creating a translational reporter construct would also be useful in determining the subcellular localization of the Y32G9A.8 protein; the reporter gene that was created was a transcriptional reporter, so localization of the protein could not be determined using this construct.

Results from earlier RNAi experiments indicate that this gene is involved in coordinating normal movement in *C. elegans*. This would indicate that the gene is involved in the nervous system. Because Down Syndrome (DS) in humans is characterized by mental retardation, clearly a defect in the nervous system, these results support the hypothesis that this gene is related to human DSCAMs. Down Syndrome in humans has several congenital gut diseases that are associated with it. This might help explain the expression pattern that was observed in *C. elegans* following injection with a reporter gene. Hirschsprung disease, characteristic of individuals with Down Syndrome, is a congenital disease in which there are no enteric ganglia along an extended length of the intestine (26). Perhaps a better understanding of this gene in *C. elegans* will lead to advanced treatments for DS-related diseases in humans.

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