Understanding How \textit{bus-1} is Regulated at the Molecular Level

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Senior Research Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in Biology in the Arts and Sciences undergraduate college of The Ohio State University

The Ohio State University, April 22\textsuperscript{nd}, 2015
Abstract

The nematode species Caenorhabditis elegans is especially suitable for studying a range of genetic and biological questions. Here, we have used C. elegans to study host/pathogen interactions. Screens of the C. elegans genome have revealed bus-1 as a gene whose product affects the nematode’s sensitivity to the bacterial pathogen Microbacterium nematophilum (Gravato-Nobre and Hodgkin, 2006). Mutations in bus-1 prevent M. nematophilum attachment and subsequent post-anal swelling in C. elegans. We aim to learn how bus-1 is regulated at the molecular level by transcription factor EGL-38 to influence expression of phenotypes in the anal region. In 2013, a colleague Benjamin Kaumeyer made a bus-1 reporter construct to determine its expression pattern. This was done by cloning a 1500 base pair fragment of DNA located 5’ to the start of the bus-1 gene into a GFP reporter. We have conducted a deletion analysis of Kaumeyer’s construct by removing four DNA fragments of various lengths upstream of the bus-1 gene to learn what region of the bus-1 promoter is regulated by EGL-38. The deletion clones were purified and injected into worms to reveal a 274 nucleotide potential EGL-38 binding site on bus-1.

Introduction

C. elegans as a model organism

C. elegans nematodes (Figure 1) are easy to breed, have relatively short life cycles, and their genetic analysis is fairly straightforward. They offer insight into many biological areas of study including cancer biology, genetics, organogenesis, molecular and cellular processes and the focus of this study, host-pathogen interactions. Many may wonder how research on a free-living soil nematode could be relevant or significant to human populations.

Significance

C. elegans nematodes are completely characterized and have essentially invariant somatic cell lineage (Kirienko et al., 2010). This consistency allows researchers to mutate its genome and study the effects and has contributed knowledge for the creation of
WormBase, a large catalog of studied, characterized, and mapped mutant strains. *C. elegans* also allow for the direct visualization of all cells, including their divisions and movements (Kirienko et al., 2010), which is important to those studying organogenesis.

Genetic mechanisms are the same in many organisms due to highly conserved domains. Among conserved genes, *C. elegans* shares 69.3% and 49.1% amino acid similarity and identity with humans, respectively. Average nucleotide identity between humans and nematodes is 49.8% among conserved genes (Wheelan et al., 1999). It is also known that about 75% of human disease genes have potential *C. elegans* homologs and 40 - 50% have *C. elegans* orthologs (Pandey and Nichols, 2011). With the underlying basis of cancer being dysfunction in a defined set of biological activities, this knowledge of the *C. elegans* genome can be applied to cancer biology in more complex organisms.

All multicellular organisms encounter bacterial pathogens and must rely on antibacterial agents, immune defenses and/or physical barriers to prevent infection and illness. While complex organisms such as humans have evolved sophisticated antigen specific, adaptive immune responses, simple organisms such as plants and *C. elegans* utilize nonspecific, innate defense mechanisms. As of late, *C. elegans* has become increasingly studied from the perspective of innate immunity because it is a species with a global distribution that lives by eating a variety of bacteria in its diet and daily life (Gravato-Nobre et al., 2005). Studies have identified a variety of different defense responses in the nematode (Darby et al., 1999; Kim et al., 2002, 2004; Huffman et al., 2004; Nicholas and Hodgkin, 2004). Thus far, most of the bacteria that have been examined with respect to pathogenic or toxic effects on *C. elegans* have been microbes known to have damaging effects on a variety of different metazoan organisms including humans, such as *Bacillus thuringiensis, Pseudomonas aeruginosa, Salmonella enterica, Serratia marcescens,* and *Staphylococcus aureus* (Mahajan-Miklos et al., 1999; Aballay et al., 2000; Labrousse et al., 2000; Marroquin et al., 2000; Couillault and Ewbank, 2002; Mallo et al., 2002; Sifri et al., 2003).

### *C. elegans/M. nematophilum* interaction

In this study, we focus on one nematode specific pathogen, *Microbacterium nematophilum*, which results in striking morphological deformation induced in the tail of infected *C. elegans* (Hodgkin et al., 2000). The bacterium is slow growing in feeding lawns of *Escherichia coli* and able to infect *C. elegans* by establishing a colony in the lumen between K, K’, F and U cells which adhere tightly to the rectal and post-anal cuticle (Figure 2) (Stiernagle, 1999). In response to the infection, the anal region of the nematode becomes greatly enlarged (Figure 3), creating a distorted morphology that is easily scored by dissecting microscope (Gravato-Nobre et al., 2005). Nematodes also become somewhat constipated and grow more slowly, but generally do not experience more deleterious effects (Gravato-Nobre et al., 2005).
Figure 2. A diagram of the L1 larval stage *C. elegans* hindgut composed of rectal epithelial cells arranged into three concentric rings (genetics.org).

Figure 3. Infection of wild-type and resistant worms. A–F show the tail region of adult hermaphrodites. (A) Uninfected wild type. (B) Infected wild type with weak Dar phenotype. Adherent bacteria are visible (arrow). (C) Infected wild type with strong Dar phenotype. (D) Infected *bus-1*, Bus phenotype. (E) Infected *bus-12*, Bus phenotype. (F) Infected *bus-12* with weak Dar phenotype, seen in a minority of animals (Gravato-Nobre *et al.*, 2005).
Establishing the relationship between *egl-38* and *bus-1*

It is known that the PAX gene family encodes transcription factors that influence cellular differentiation in uterine and tail development. The EGL-38 protein is an ortholog of mammalian class proteins PAX2/5/8 (Chamberlin et al., 1997) and is important for the development of the *C. elegans* hindgut, exclusively where we see swelling in response to interaction with *M. nematophilum*. The morphology and function of the hindgut cells can be disrupted by loss of function mutations which include *egl-38, mab-9* and *mab-23* (Chisholm and Hodgkin, 1989; Chamberlin et al., 1997; Woollard and Hodgkin, 2000). When a gene expression microarray comparison was conducted between wild type and said mutant *egl-38* nematodes, 15 of 33 genes whose expression alters based on the presence of *egl-38* were found to encode proteins important in fatty acid biosynthesis (Table 1). We focus on these 15 genes because it is believed that the formation of lipid rafts plays a role in *M. nematophilum* sticking to the lumen between anal/tail region cells. A study completed by Gravato-Nobre et al. revealed that 121 mutant nematodes showed an altered response to infection and demonstrated bacterially unwollen (Bus) phenotypes when using both chemical and transposon mutagenesis (Gravato-Nobre et al., 2005). Some of the mutants correspond to known genes while most defined 15 new genes including *bus-1*, which encodes an integral membrane O-acyltransferase.

**Table 1.** Five of the fifteen fatty acid biosynthesis genes that are altered in expression in *egl-38* mutants. The values represent the ratio of mRNA transcript abundance in the indicated *egl-38* mutant compared to the abundance in wild type animals.

<table>
<thead>
<tr>
<th>gene id</th>
<th>gene name</th>
<th>encoded protein</th>
<th>egl-38(n578)</th>
<th>egl-38(sy294)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R09B5.6</td>
<td>had-1</td>
<td>hydroxy-Acyl-CoA Dehydrogenase</td>
<td>0.428</td>
<td>0.410</td>
</tr>
<tr>
<td>Y105E8A.4</td>
<td>ech-7</td>
<td>enoyl-CoA hydratase</td>
<td>0.493</td>
<td>0.537</td>
</tr>
<tr>
<td>Y67A10A.1</td>
<td>oac-56</td>
<td>membrane O-acyltransferase</td>
<td>0.343</td>
<td>0.371</td>
</tr>
<tr>
<td>C36A4.9</td>
<td>acs-19</td>
<td>Acyl-CoA synthetase</td>
<td>0.466</td>
<td>0.486</td>
</tr>
<tr>
<td>R03H4.6</td>
<td>bus-1</td>
<td>membrane O-acyltransferase</td>
<td>0.296</td>
<td>0.272</td>
</tr>
</tbody>
</table>

Ratio of mRNA abundance in the mutant *egl-38* to wild type *egl-38*. 

*Wild type fatty acid biosynthesis*
The microarray data shows that mutant egl-38 leads to lowered fatty acid biosynthesis gene expression and that wild type egl-38 promotes expression. We conclude that bus-1 is dependent on egl-38 (Table 1) and set the main objective to learn what region of the bus-1 promoter is regulated at the molecular level by EGL-38. Recombinant DNA technology has revealed that, consistent with the gene’s function in hindgut establishment and maintenance, bus-1::GFP reporter fusion is expressed in rectal cells from late embryogenesis through adulthood in the rectal epithelium (Gravato-Nobre and Hodgkin, 2008). The specific expression pattern makes bus-1 a valuable hindgut marker for morphological and developmental studies, and in particular, in situations where the shape and the dimension of the rectal epithelial cells are to be inspected (Gravato-Nobre and Hodgkin, 2006).

Materials and Methods

Maintaining C. elegans

C. elegans nematodes are maintained on Nematode Growth Medium (NGM) agar petri plates at 20°C. E. coli is used as a bacterial food source to promote growth and reproduction of healthy nematodes. These plates, abundant with nematodes, are then useful for mutant screening of bus-1::GFP reporter constructs.

Making the bus-1::GFP reporter constructs via PCR

The bus-1::GFP reporter constructs were created using two methods, only one of which yielded the best results. The goal of the first method was to amplify three different sized regions within the 1500 base pair located 5’ to the start of the bus-1 gene, fuse the fragment upstream of a GFP coding sequence on a plasmid, pPD95.69 and then observe fluorescence patterns in nematodes. Three PCR reactions were run using purified C. elegans N2 genomic DNA as a template, one 20µM reverse primer and three different 20µM forward primers each engineered with XbaI and BamHI restriction sites. Additional buffers, dNTPs and water were added to the reaction mixtures according to the NEB M0273S PCR Protocol. Gel electrophoresis of the three PCR products yielded the expected 1.2 kB, 1.3 kB and 1.4 kB fragments. These products and pPD95.69, a dephosphorylated GFP vector with CARB1 gene and one XbaI and BamHI restriction sites, were digested with XbaI and BamHI at 37°C and the products purified. Following overnight ligation of the PCR fragments and pPD95.69 at 15°C, the products were transformed into competent DH5α E. coli cells, plated on LB+ CARB and incubated at 37°C overnight. Extracted plasmids were purified and diagnostic restriction digests were used to ensure accuracy of this first, PCR cloning method. Four weeks of repeated diagnostic restriction enzyme digests with BamHI and XbaI showed that the recombinant vector was not re-isolating into the empty pPD95.69 and PCR fragments following gel electrophoresis. This demonstrated the possibility that the ligation of the two and, thus this method, were not successful. Alternatively, we tried using PvuII, which cuts the pPD95.69 vector twice, to conduct diagnostic digests. We hypothesized that it would more efficiently yield two distinct bands of the PCR fragments and empty pPD95.69 vector. Repeated diagnostic digests with PvuII yielded two bands as predicted and appeared to be
successful; however, sequencing results showed that the PCR inserts was not present in any of the three recombinant pPD95.69 vectors we engineered.

Making the bus-1::GFP reporter constructs via deletion cloning

Deletion cloning used pBK1 as a template to obtain the same end goal: identification of the bus-1 binding sequence where EGL-38 regulates the gene. In 2013, Benjamin Kaumeyer made pBK1, a bus-1 reporter construct, to confirm its expression pattern. Again, this was done by cloning the 1500 base pair fragment of DNA located 5’ to the start of the bus-1 gene into a plasmid containing a GFP coding sequence, CARB’ gene and Xbal and BamHI restriction sites. Benjamin conducted deletion cloning on pBK1 by cutting at five sites upstream of the bus-1 start codon, making subsequently smaller regions (Figure 4). He observed that between the fourth and fifth smallest fragments GFP expression in nematodes changed from positive to negative (Figure 5).

![Figure 4](image)

**Figure 4.** Benjamin Kaumeyer’s DNA fragments upstream of the bus-1 gene that were used to drive expression of GFP in the deletion analysis bus-1 reporter constructs are represented by the blue bars. The X-axis represents the location X number of nucleotides upstream of the start codon of the bus-1 gene. The coding sequence of bus-1 is to the right of 0. Reporter construct H fuses together the two fragments connected by the line.

![Figure 5](image)

**Figure 5.** Benjamin Kaumeyer’s results from deletion analysis. The Y-axis represents the proportion of animals showing expression in either K, K’, U or F. The X-axis represents the number of nucleotides upstream of the start codon of the bus-1 gene. Each point represents the proportion of animals containing the deletion reporter construct referenced in figure 9. The X value represents the location X nucleotides upstream of the start codon that drives expression of the GFP reporter.

We hypothesize that by reintroducing an Xbal cutting site upstream of the bus-1 start codon in pBK1 at four additional sites, between Benjamin’s F and G fragments, we would again yield positive or negative GFP expression in nematodes and narrow down on the specific nucleotide sequence where EGL-38 binds to regulate the gene. We designed one reverse primer and four forward primers each with Xbal sites to amplify four
different sized fragments. The first primer yielded an upstream DNA fragment of 752 nucleotides, the second 529 nucleotides, the third 404 nucleotides and the fourth 130 nucleotides (Figure 6).

![Figure 6](image)

**Figure 6.** DNA fragments upstream of the bus-1 gene that were used to drive expression of GFP in the deletion analysis bus-1 reporter constructs are represented by the blue bars. These fragments fall within those created by Benjamin Kaumeyer, years ago. The X-axis represents the location X number of nucleotides upstream of the start codon of the bus-1 gene. The coding sequence of bus-1 is to the right of 0.

Following the NEB M0530 PCR Protocol for Phusion, four PCR reactions were run using pBK1 as a template, the reverse and forward primers, dNTPs, water and Phusion Buffer and Phusion Enzyme. The products were washed with Buffer PE to remove excess salts and digested overnight at 37°C with restriction enzymes DpnI and XbaI. The products were ligated at 15°C overnight then transformed into competent DH5α *E. coli* cells, plated on LB+ CARB and incubated again at 37°C overnight. Extracted plasmids were purified and diagnostic BanII restriction digests were used to ensure the E1 and E2 fragments were obtained and PvuII was used to ensure the E3 and E4 fragments were obtained. The diagnostic digests showed that the deletion cloning method yielded all four of the expected fragments following eight weeks of experimentation. Sequencing also confirmed that the deletion cloning method was successful.

E3 and E4 vectors were injected into a wild type *C. elegans* nematode growing on NGM agar petri plates at 20°C with *E. coli*. Three lines with 28 total E3 nematodes and six lines with 268 total E4 nematodes were established. We defined an established line of progeny as motile and well fed nematodes. Starved, oversized and deformed nematodes were not scored for GFP expression. Using a fluorescence scope over a period of 5 weeks, normal E3 and E4 progeny were scored for hindgut cells with GFP fluorescence. Those progeny with above background level GFP were considered GFP+.
Results

Sequencing and BLAST

The results of the deletion cloning method reveal that it was indeed the most effective approach to learning more about the regulatory relationship between EGL-38 and *bus-I*. The genes and transcription factors responsible for normal hindgut development in response to *M. nematophilum* are known and mutants that alter its development continue to be extensively studied. The specific effects of mutating *bus-I* were examined in this study to determine where the transcription factor EGL-38 finds the correct DNA sequence to control *bus-I*. In other words, we aimed to find the EGL-38 binding site on the *bus-I* gene.

The four mutants upstream of *bus-I* in the pBK1 plasmid, were 752, 529, 404 and 130 nucleotides in length. The sequence of the mutants was confirmed by running a nucleotide BLAST of the query, mutant pBK1 and subject, wild type pBK1. The four mutants, Experimental 1 (E1), Experimental 2 (E2), Experimental 3 (E3) and Experimental 4 (E4), showed 100%, 99%, 94% and 97% identities, respectively (Figure 7-10). Three E3 and three E4 transgenic lines which resulted from injection show that a ratio of normal progeny to GFP+ normal progeny was 28:17 (61%) and 32:0 (0%) (Figure 11).

![Figure 7. A section of the E1 (query) and wild type pBK1 (subject) sequencing results using BLAST nucleotide.](image)
Figure 8. A section of the E2 (query) and wild type pBK1 (subject) sequencing results using BLAST nucleotide.

Figure 9. A section of the E3 (query) and wild type pBK1 (subject) sequencing results using BLAST nucleotide.

Figure 10. A section of the E4 (query) and wild type pBK1 (subject) sequencing results using BLAST nucleotide.
Potential *bus-1* promoter binding region

The work of Benjamin Kaumeyer showed that between his F and G deletion clones of pBK1, transgenic lines went from GFP- to GFP+ (Figure 5). By designing four clones with slight differences in the number of nucleotides and that fall between his F and G fragments, we have been able to narrow down the EGL-38 binding site on the *bus-1* gene. Because E3 was GFP+ and E4 was GFP- we can predict that injection of E2 will be GFP+. Thus the EGL-38 binding site on the *bus-1* gene is within the 274 nucleotide sequence that was removed when E4 was engineered relative to E3 (Figure 6). Previous studies confirm that sequences of mutant *bus-1* longer than E2 were GFP+ and thus the prediction that E2 is GFP+ is highly probable.

In short, the swelling response in the hindgut of *C. elegans* nematodes when exposed to the bacterial pathogen *M. nematophilum* provides a framework for studying host pathogen interactions. The results of the deletion cloning method help us better understand the developmental and regulator gene network between EGL-38 and *bus-1* which influences the swollen or unswollen response in *C. elegans* when interacting with the nematode specific pathogen.

**Discussion**

**Significance of our findings**

We hypothesize that within the 274 nucleotide sequence identified on the mutant *bus-1*, EGL-38 binds to regulate the gene. This finding is significant because *bus-1* is strongly expressed in the rectal epithelial cells and renders the nematode susceptible to infection by *M. nematophilum* (Gravato-Nobre and Hodgkin, 2008). Additionally, exposure of the
tissue specific pathogen to mutant *C. elegans* will provide an effective way of gaining a deeper understanding of regional specialization, which might be otherwise undetectable (Gravato-Nobre and Hodgkin, 2008).

Entering the 274 nucleotide sequence in Jasper revealed that in addition to the hypothesized EGL-38 protein, five PAX2 proteins found in *Homo sapiens* and *Mus musculus* have confirmed binding sites within the nucleotide sequence (Figure 12). Thus, we know that identification of the 274 nucleotide sequence not only provides an effective way to study how EGL-38 regulates expression of *bus-1* gene products but also study vertebrates.

Our results also show that *bus-1* expression is not entirely lost in mutant *egl-38* nematodes. So it is unlikely that lowered *bus-1* expression fully explains resistance to *M. nematophilum*. There is the possibility that EGL-38 controls a battery of genes in rectal epithelial that *M. nematophilum* can specifically recognize. To test this, a *bus-1* rescue construct was made by colleague Ben Kaumeyer (Figure 13).

**Figure 12.** Five PAX2 proteins known to have binding sites on the 274 nucleotide sequence we identified and engineered in this study (jaspar.genereg.net).

**Figure 13.** The proportion of animals displaying a DAR phenotype after developing on mixed lawns of OP50 and *M. nematophilum*.
Future Work

From the 274 nucleotide sequence we could further narrow down the EGL-38 binding site by creating fragments between E3 and E4 (Figure 6). In vitro experimentation could also be conducted to see where EGL-38 binds. Exposing transgenic progeny to *M. nematophilum* would confirm the work presented here and aid researchers studying the tissue specific expression of *bus-1*. Because the sequence has confirmed binding sites for PAX2 proteins, this study can also be tested on more complex organisms and eventually be used to learn about development in humans.

References


