

Using RNA-Mediated Interference to Investigate the Role of Novel Proteins in *Caenorhabditis elegans* Gonadogenesis

Georgette N. Jones

Faculty Sponsor: Jennifer Miskowski, Department of Biology

ABSTRACT

During development, cells divide, differentiate into various cell types, and organize into functional tissues and organs. We are interested in understanding the cellular and molecular basis of organ formation in the nematode *Caenorhabditis elegans*. To this end, we are investigating two novel proteins that have a role in gonadogenesis, SYS-1 and NUD-1. SYS-1 was identified via a genetic screen for mutants with defective gonads. The role of SYS-1 at the cellular level has been characterized (1), but its molecular role is unclear. NUD-1 is homologous to a protein in fungi responsible for mediating nuclear migration (2). Using reverse genetics to “knock-down” the protein levels, *C. elegans* NUD-1 was shown to have a role in gonadogenesis, but the specific phenotype has not been described in detail. We are using RNA mediated interference (RNAi) to investigate both of these proteins. RNAi is a method by which adding an excess of mRNA corresponding to a specific gene actually represses the production of that protein. Information learned about gonad formation in worms is likely to advance our understanding of organ development in more complex organisms.

INTRODUCTION

The mechanisms by which cells divide, differentiate, migrate, and die during development are very important and interesting, but they are not fully understood. A complex series of processes is required for proper development, and it is essential that they be precisely coordinated to form a normal functioning organism. Likewise, a combination of multiple cellular processes is needed for proper formation of an organ. The molecular basis of organ formation is the basis for this study.

A model system for studying organ development: the Caenorhabditis elegans gonad

In order to study organ development, *Caenorhabditis elegans*, a 1mm long non-parasitic soil nematode, was employed as a model organism. *C. elegans* proves to be a good model system for investigating these highly conserved processes due to its genetic and molecular similarity to higher organisms, such as humans. In addition, these worms are easy to grow, they have a short lifespan, and their genome has been entirely sequenced. They are small and transparent which allows them to be examined at the level of individual cells, and the development of all of their somatic cells is essentially constant from animal to animal. *C. elegans* has often been used to study organ development, and this study focuses specifically on the role of two proteins, NUD-1 and SYS-1, in the development of the hermaphrodite gonad.

The *C. elegans* hermaphrodite gonad is a complex organ with distinct cell-types and functional tissues (3). There are four larval stages of development (L1-L4) of a worm before it becomes an adult. When a worm hatches as an L1, the football-shaped gonad consists of only four cells referred to as the gonad primordium. These are the cells that will ultimately form the adult gonad. Of these four cells, two are somatic precursors (Z1 and Z4) and two are germ cells (Z2 and Z3). The somatic precursors will form the various cells/tissues that house the germ cells and support their development. The somatic gonadal cells develop in a nearly identical manner in all hermaphrodites allowing the processes to be studied at the cellular level. From L1-L4, the gonad adopts the U-shaped morphology of the adult organ and gives rise to the mature gonad tissues such as the spermathecae, uterus, and sheath. It has been shown that the SYS-1 and NUD-1 proteins are necessary for gonad formation to occur properly (1,2).

The proteins of interest: SYS-1 and NUD-1

SYS-1 and NUD-1 proteins were identified using distinct approaches, but were both found to be involved in gonad formation. SYS-1 was discovered using a forward genetics approach. Forward genetics is a process by

which worms are subjected to various mutagens and mutant worms with the desired phenotype are selected for study. The mutated gene (and therefore, protein) responsible for the phenotype is then identified and examined. SYS-1 has been found to be part of an evolutionarily conserved signal transduction pathway called the Wnt pathway (4). In contrast, NUD-1 was identified by reverse genetics. Here, a protein sequence that has been identified and studied in another organism is searched against a database containing the worm's entire genomic sequence. If a protein with similar sequence (homologous protein) is found, its function can then be investigated in worms. NUD-1 was known to play a role in nuclear migration in several other organisms from fungi to mammals, and a related protein was identified in *C. elegans* (2). Though the genes were discovered using different methods, a single approach, termed RNA mediated interference (RNAi), was used in this study to understand the functions of the protein products.

The process: RNA-mediated interference (RNAi)

RNAi is a method by which the introduction of large amounts of mRNA for a specific gene, either by injection, soaking, or feeding, actually represses the production of the corresponding protein. The high concentration of exogenous mRNA added to the animals leads to the degradation of the endogenous mRNA, which is the template for making the protein. This method was employed to significantly "knock-down" the levels of the proteins of interest to ultimately determine the molecular function of the two proteins. By examining the development of an organism in the absence of a particular protein, the function of that protein can be determined. The physical outcome, or phenotype, of an organism that has developed in the absence of a particular protein can shed light on the normal function of the protein itself. Once the functions of all the proteins within a pathway are understood, the "pieces" can be put together and a general understanding of a developmental process is possible.

MATERIALS AND METHODS

Polymerase chain reaction to amplify the C. briggsae sys-1 gene

Polymerase chain reaction (PCR) was performed to specifically amplify the *C. briggsae sys-1* gene for use in an *in vitro* transcription reaction (Figure 1). T7 RNA polymerase recognition sites were attached to the ends of the PCR primers so that the T7 sites would be incorporated into the PCR product.

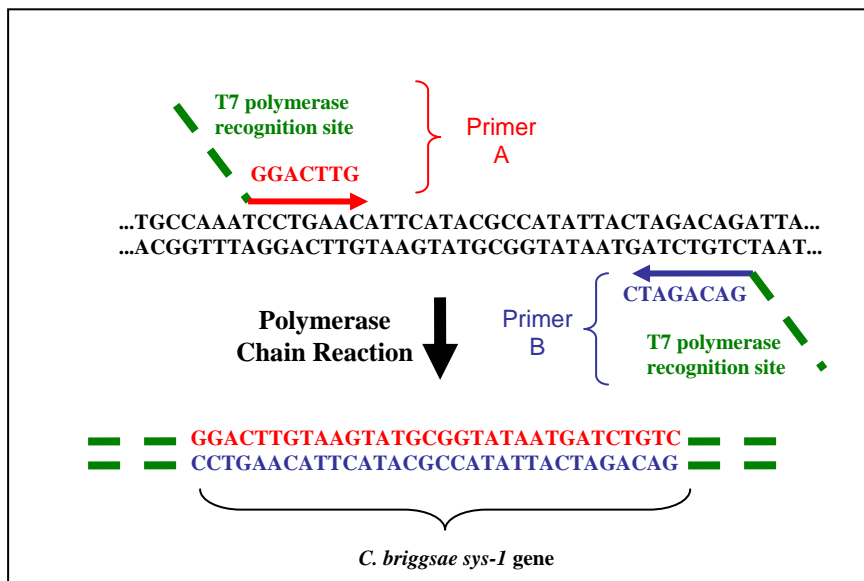


Figure 1. PCR design with primers specific for *C. briggsae sys-1* and tagged with a T7 polymerase recognition site so the PCR product could be used directly in an *in vitro* transcription reaction.

In vitro transcription to obtain C. briggsae sys-1 RNA and RNAi soaking method

The *C. briggsae sys-1* PCR product was used directly in a MEGAscript T7 *in vitro* transcription reaction (Ambion, Inc., Austin, TX). The RNA product of *in vitro* transcription was purified by LiCl precipitation and used

for RNAi via the soaking method in *C. briggsae*. With this method, adult worms were soaked in bleach and NaOH to break down their cuticles, thereby sacrificing the worms, but releasing their embryos that were protected by their eggshells. The embryos were washed several times in M9 buffer to remove the bleach and allowed to hatch into L1 worms. The L1 worms were precipitated and resuspended in a solution containing mRNA corresponding to the *C. briggsae sys-1* gene. After soaking for 36 hours, the worms were placed on agar plates with bacteria, allowed to grow to adulthood, and observed for mutant gonad phenotypes.

RNAi feeding method

The NUD-1 protein was eliminated from *C. elegans* worms via RNAi feeding by utilizing the bacteria *E. coli* to produce *nud-1* RNA in mass quantities. Since *C. elegans* worms eat *E. coli OP50* as a part of their normal diet, this method proved to be effective. Calcium competent *E. coli* HT115 cells were prepared to readily take up foreign DNA. The competent cells were transformed by heat shock with the *nud-1* cDNA contained within the inducible feeding plasmid L4440 plasmid carrying antibiotic resistance to ampicillin and tetracycline. The cells were plated on LB agar plates with the antibiotics for selection. From those plates, single colonies were grown overnight in LB broth containing ampicillin and tetracycline. The overnight bacterial growths were spiked with IPTG, which induced the transcription of the *nud-1* cDNA cloned into L4440 into RNA. Normally, the worms live on agar plates that contain all the ingredients necessary for them to grow healthily. For this experiment, those plates also contained IPTG, ampicillin and tetracycline. The bacteria which was transformed with *nud-1* cDNA, and induced to make RNA, was used to “seed” the plates. 200 μ L of the bacteria were added to each plate and served as the food source for any worms placed on those plates. Small chunks of agar from plates containing starved L1 worms arrested in development, were put on the new plates seeded with the transformed bacteria. The starved worms moved toward the bacterial food source and ingested the mass quantities of RNA. With a sufficient food supply, the worms re-entered normal development. Three days later, the worms were analyzed by dissecting microscope and differential interference contrast microscopy (DIC) at 600X magnification for malformations in the gonad.

RESULTS

Investigating the molecular basis of SYS-1 action in the gonad

To initiate a structure/function analysis of the *C. elegans* SYS-1 protein, an evolutionary approach was taken. The *C. elegans* SYS-1 protein sequence was compared to the SYS-1 protein sequence in *Caenorhabditis briggsae*, a relative of *C. elegans*. Significant similarity existed between the two proteins (data not shown). The first goal was to determine if the SYS-1 protein in *C. briggsae* has the same function as SYS-1 in *C. elegans*. If so, the regions of the SYS-1 protein that are most highly conserved between the two species would be studied further. The products from the PCR experiment as well as the *in vitro* transcription reaction were of the expected size (Figure 2). However, when used in the soaking method of RNAi, no mutant phenotypes were observed in the tested worms.

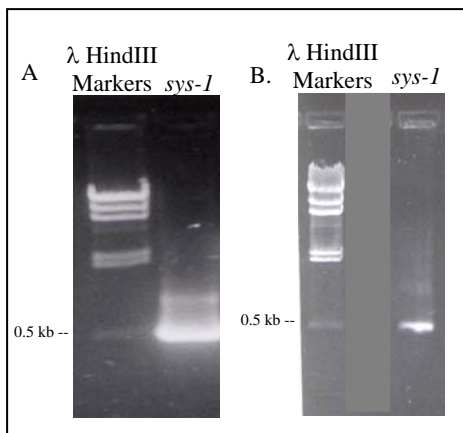


Figure 2. (A) Agarose gel electrophoresis results for PCR amplification of *sys-1*. (B) Results for *in vitro* transcription of *sys-1* RNA. The products were of the expected size (0.5 kb).

Investigating the cellular role of *NUD-1* in the gonad

Normally, the ventral center of the hermaphrodite gonad is made up of the uterus, containing young embryos, which is flanked by spermatheca on each side. In *NUD-1* mutants, this area was full of what appeared to be spermatheca rather than embryos (Figure 3). *NUD-1* RNAi mutant worms that expressed a GFP marker for spermathecal cells also supported the hypothesis that the excess tissue was spermatheca (data not shown). Note that in Figure 3 a wild-type worm has several embryos lined up within the uterus awaiting expulsion from the vulva, whereas there are little to no embryos in the *NUD-1* mutants. Also, the *NUD-1* mutants appear to lack a uterus allowing the spermathecae to inhabit the entire central ventral region of the gonad.

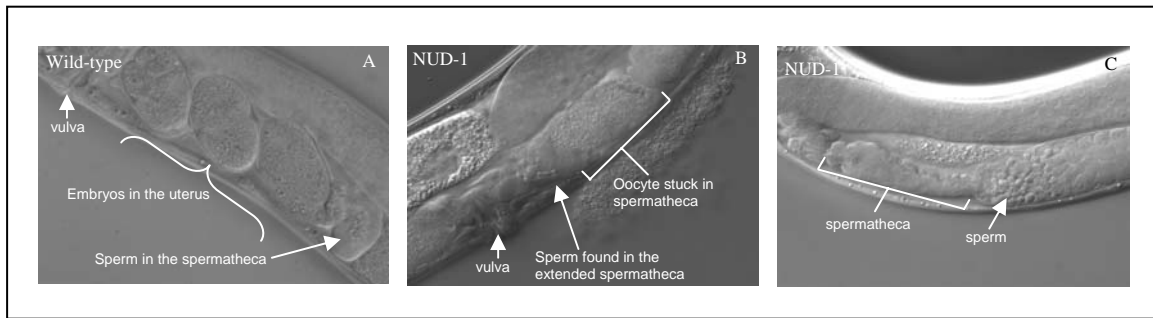


Figure 3. Gonad phenotype associated with *NUD-1* RNAi. Animals were observed with DIC microscopy at 600X magnification. (A) A wild-type hermaphrodite gonad, showing the distinct spermatheca and the uterus containing embryos. (B,C) *NUD-1* RNAi mutant worms showing extended spermathecal regions that almost reach the vulva and no fertilized embryos in the uterus.

Transgenic worms carrying a *NUD-1::GFP* (green fluorescent protein) construct were observed during early stages of development for cells that expressed *NUD-1* (Figure 4). At the early L3 larval stage, there are five uterine precursor cells, which lead to the formation of the uterus in a healthy animal. These cells are specific and are characterized by their position and arrangement in the somatic gonad primordium as two inverted triangles (one complete and one incomplete). At least a subset of the uterine precursor cells fluoresces in the transgenic worms (Figure 4), meaning that *NUD-1* is expressed. Note that the intestine also shows fluorescence. This is due to granules present in the intestinal tract that autofluoresce, and should not be compared to the cells that fluoresce in the gonad.

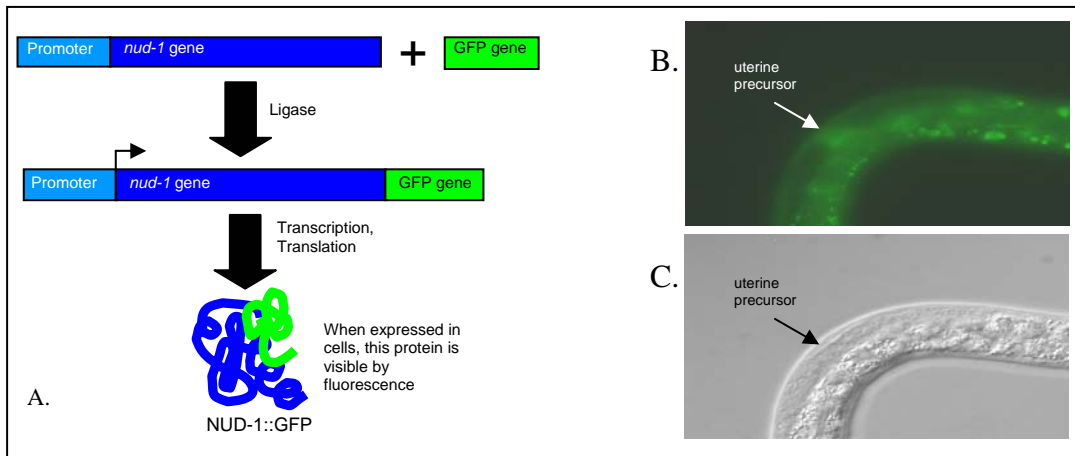


Figure 4. Schematic of *NUD-1::GFP* construct and expression in *C. elegans* gonad. (A) *NUD-1::GFP* fusion protein is made by ligating the GFP gene to the *nud-1* gene. (B) *NUD-1::GFP* expressing cells in *C. elegans* hermaphrodite gonad as viewed by fluorescence microscopy at 600X magnification. Note the expression in uterine precursor cells. (C) DIC image of the animal in (B) at 600X magnification.

DISCUSSION

The results for RNAi by soaking for *C. briggsae* SYS-1 were inconclusive as no mutant phenotypes were observed. A different method of RNAi, such as the injection method, may prove to be more effective. With the injection method, *sys-1* RNA is directly injected into the animals to elicit the same effects as RNAi by any other method.

RNAi by feeding for NUD-1 in *C. elegans* resulted in mutant worms that appeared to have extra spermatheca in the region where the uterus is usually found. Thus, there was a lack of embryos and a normal uterus in the mutant worms. NUD-1::GFP worms showed fluorescence in uterine precursor cells, thus supporting the hypothesis that NUD-1 may play a role in uterus formation. Further work is underway to analyze the NUD-1 RNAi phenotype in more detail, and a genetic screen will be conducted to try and obtain a heritable mutation in the *nud-1* gene. A nuclear-localized version of the NUD-1::GFP construct will be made in order to better pinpoint the expression pattern. A genetic and molecular approach will be used to try and understand the mechanism by which NUD-1 functions in the *C. elegans* gonad.

LIMITATIONS

Limitations for this project were found primarily with the SYS-1 experiments. Without the necessary equipment or funding, the injection method of RNAi for SYS-1 was not possible. Therefore, the soaking method was the only possibility for this project and the results were inconclusive.

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REFERENCES

1. Miskowski, J.A., Li, Y., and Kimble, J.E., *Dev. Biol* **230**, 61 (2001).
2. Dawe, A.L., Caldwell, K.A., Harris, P.M., Morris, N.R., and Caldwell, G.A., *Dev Genes Evol* **211**, 434 (2001).
3. Kimble, J. and Hirsh, D. *Dev. Biol* **70**, 396 (1979).
4. Siegfried, K.R. and Kimble, J. *Development* **129**, 443 (2002).