Biol 322 Fall 2012
RNAi, Reverse Genetics and Targeted knockdown of gene function

In this lab exercise, you will employ RNAi to knock down the function of specific genes in C. elegans. The double stranded RNA will be delivered to the worms via E. coli containing plasmids expressing dsRNA (double stranded) constructs. dsRNA complementary to two genes, dpy-11 (dumpy) and bli-1 (blister) will be fed to wild type worms, and the phenotypes compared in RNAi treated worms, and worms with loss-of-function mutations at the same loci.

1. Introduction to reverse genetics in *C. elegans (from the wormbook.org)*

Through genetic analyses, the function of genes is investigated by studying organisms where gene function is altered. In classical forward genetic screening, individuals are treated with mutagens to induce DNA lesions and mutants with a phenotype of interest are sought. After a mutant is found, the gene mutated is identified through standard molecular techniques. Detailed studies of the mutant phenotype coupled with molecular analyses of the gene allows elucidation of the gene's function. Forward genetics has been responsible for our understanding of many biological processes and is an excellent method for identifying genes that function in a particular process.

In reverse genetics, the functional study of a gene starts with the gene sequence rather than a mutant phenotype. Using various techniques, a gene's function is altered and the effect on the development or behaviour of the organism is analysed. Reverse genetics is an important complement to forward genetics. For example, using reverse genetics, one can investigate the function of all genes in a gene family, something not easily done with forward genetics. Further, one can study the function of a gene found to be involved in a process of interest in another organism, but for which no forward genetic mutants have yet been identified. Finally, the vast majority of genes have not yet been mutated in most organisms and reverse genetics allows their study. The availability of complete genome sequences combined with reverse genetics can allow every gene to be studied.

This (reverse genetics) chapter gives detailed protocols for the two main methods of perturbing gene function in C. elegans: RNA interference and the creation of deletion mutants. Either technique can be applied to the study of individual genes. With less than a day of actual work, RNAi creates a knockdown of gene function without altering the organism's DNA/ In contrast, with about a month of work, a deletion mutation permanently removes all gene function. Deciding which technique to use will depend on the nature of the experiment. The techniques can also be combined, where RNAi is used for rapid screening of loss of function phenotypes and then deletion mutants are made to study genes of particular interest. RNAi can also be carried out on a global scale, where knockdown of (nearly) every gene is tested for inducing a phenotype of interest. In this case, the reverse genetics technique of RNAi can be thought of as a forward genetic screening tool.

RNAi (RNA interference) refers to the introduction of homologous double stranded RNA (dsRNA) to specifically target a gene's product, resulting in null or hypomorphic phenotypes. Gene knockdown via RNAi is ***highly specific***, ***remarkably potent*** (only a few dsRNA molecules per cell are required for effective interference) and ***the interfering activity*** (and presumably the dsRNA) can cause interference in cells and tissues ***far removed from the site of introduction.***

Check out this RNAi animation:
http://www.nature.com/focus/rnai/animations/index.html

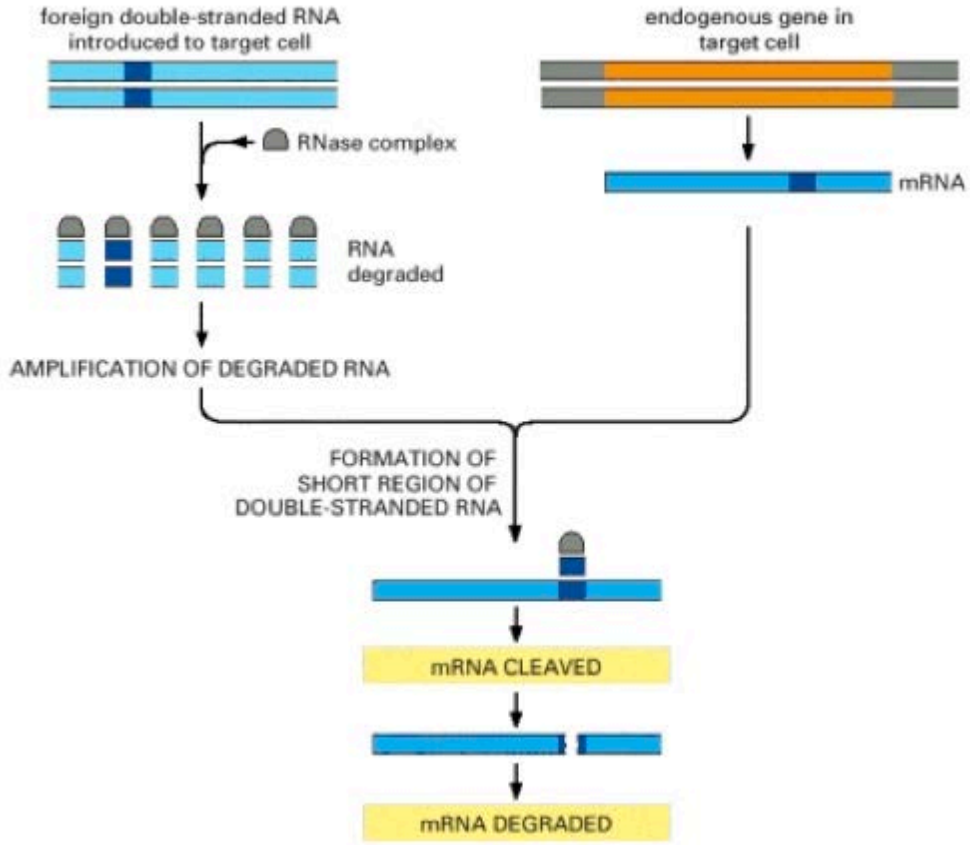

Figure 7-107 Molecular Biology of the Cell 2002

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The mechanism of RNA interference On the left is shown the fate of foreign double-stranded RNA molecules. They are recognized by an RNase, present in a large protein complex, and degraded into short fragments that are approximately 23 nucleotide pairs in length. These fragments are sometimes amplified by an RNA-dependent RNA polymerase and, in this case, can be efficiently transmitted to progeny cells. If the foreign RNA has a nucleotide sequence similar to that of a cellular gene (*right* side of figure), mRNA produced by this gene will also be degraded, by the pathway shown. In this way, the expression of a cellular gene can be experimentally shut off by introducing double-stranded RNA into the cell that matches the nucleotide sequence of the gene. RNA interference also requires ATP hydrolysis and RNA helicases, probably to produce single-stranded RNA molecules that can form base pairs with additional RNA molecules.

Introducing DS-RNA into an organism

There are three ways to carry out RNAi gene knockdown in C. elegans:

1. **RNAi by injection:** dsRNA produced in vitro is injected into young adult hermaphrodites and their progeny scored for mutant phenotypes.
2. **RNAi by soaking:** worms are soaked in a high concentration dsRNA solution and then subsequently they or their progeny scored for phenotypes.
3. **RNAi by feeding:** *E. coli* producing the desired dsRNA are fed to worms and either they or their progeny are scored. Worms of any stage can be subjected to RNAi by feeding.
4.

E. Coli **Strains used for this experiment**

HT115 refers to the strain of E. coli – it is defective in RNaseIII (specifically binds and cleaves double-stranded RNA
pL440 is the "feeding" vector (see map on next page)

HT115(DE3)/pL440(*bli-1*) – vector carries a segment of the ***bli-1 gene***

HT115(DE3)/pL440(*dpy-11*) - vector carries a segment of the ***dpy-11 gene***

HT115(DE3)/pL440(empty) - RNAi feeding vector (control) – plasmid contains no *C. elegans* sequences

C. elegans **Strains:**

wildtype
dpy-11(e224)V – dumpy reference mutant strain
bli-1(e769)II – blister reference mutant strain

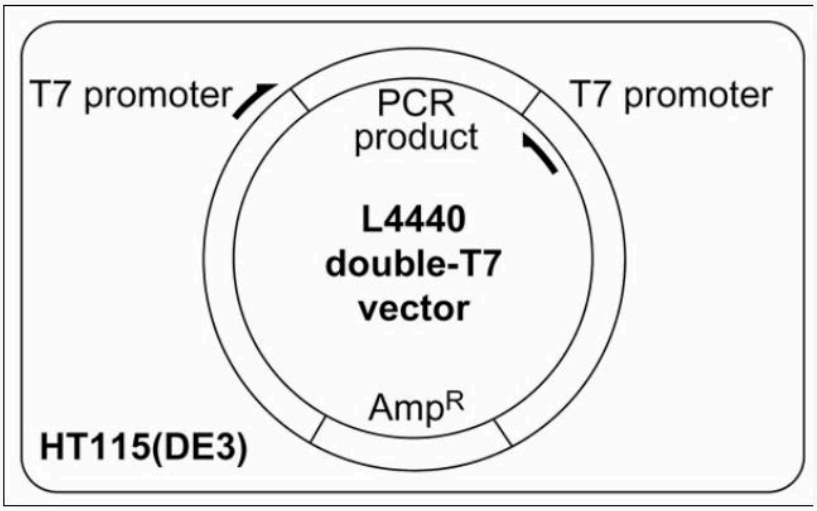


Figure 1

L4440 double-T7 vector inside HT115 RNase-deficient *E. coli*. A fragment from the gene of interest is amplified by PCR and cloned into the L4440 double-T7 vector, which has two T7 promoters in inverted orientation flanking the multiple cloning site [4]. Cloned plasmids are transformed into HT115(DE3), an RNase III-deficient *E. coli* strain with IPTG-inducible expression of T7 polymerase (L. Timmons and A. Fire, personal communication).

Genome Biol. 2001; 2(1): research0002.1–research0002.10.

T7 promoter = promoter sequences lifted from the genome of the T7 E. coli bacteriophage

Transcription of the *C. elegans* sequences (PCR product in above map) can proceed using both strands as template because there are two T7 promoters flanking the sequence oriented on oppposite strands.

The double-strand RNA is synthesized only when T7 RNA polymerase is produced in the cell. Its transcription is (artificially) under the control of the lac repressor protein which in turn is under allosteric control by the compound IPTG.

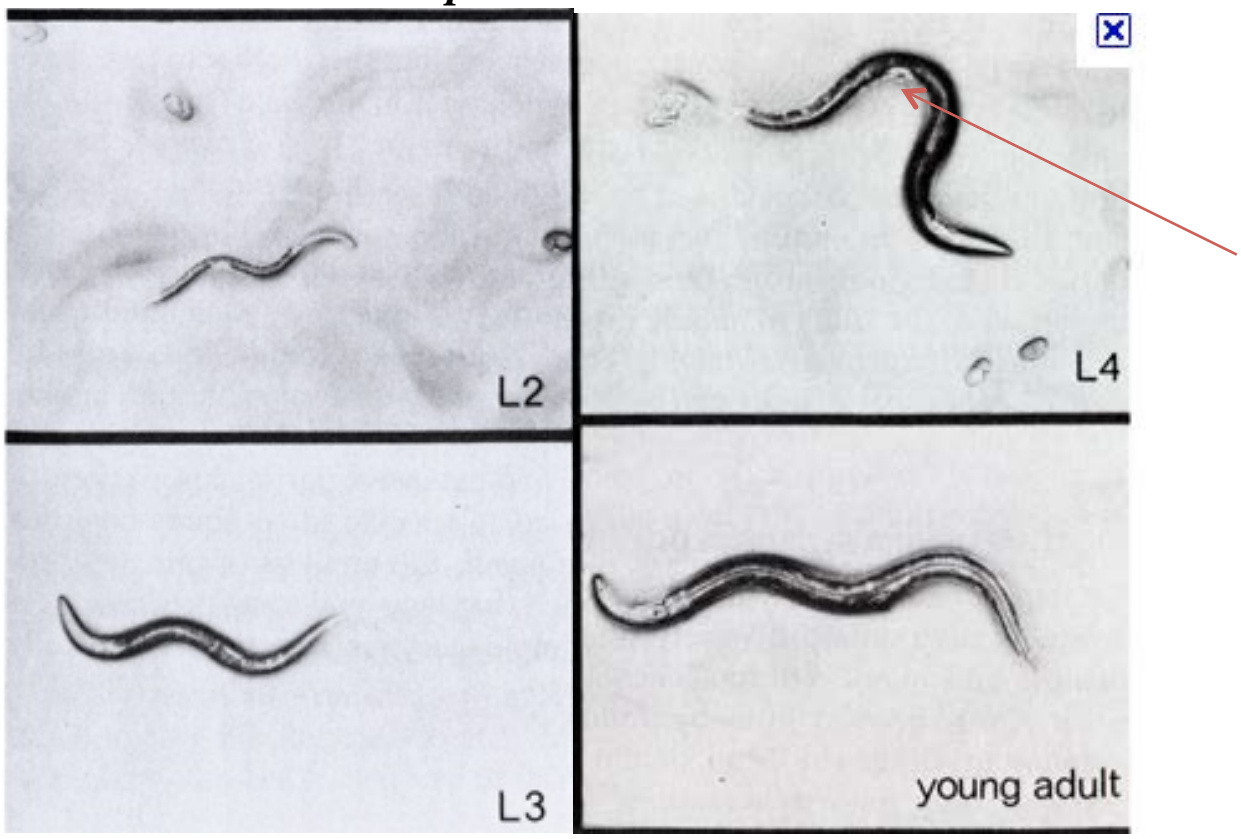
Protocol Starts Here:

STEPs 1-6 will be done for you – you do not need to include each step in your google doc form – just summarize

1. Streak *E. coli* strains to single colony on LB +Ampicillin (100μg/mL) + Tetracycline (100μg/mL) plates. Incubate at 37°C overnight.
2. For each *E. coli* strain, inoculate one colony to flask with 100mL LB broth + Ampicillin (100μg/mL). Shake at 37°C overnight, 2000 rpm.
3. For each *E. coli* strain, transfer 4mL of overnight culture to flask with 100mL LB broth + Ampicillin (100μg/mL). Shake at 37°C, 2000 rpm, for 2 hours (approximately 0.2 – 0.6 OD for semi-log phase).

4. Add IPTG (1mM) to each flask to induce dsRNA transcription. Shake at 37°C, 2000 rpm, for 4 hours.
5. To concentrate cells, centrifuge cultures at 3000 rpm for 10 minutes. Resuspend each culture in 4mL M9 buffer with Ampicillin (100μg/mL) and IPTG (1mM).
6. For each culture, aliquot 250μL to NGM-lite +Ampicillin (100μg/mL) + IPTG (0.4mM) plates. Let plates dry at room temperature overnight.

Experiment Starts Here



Tuesday Nov. 27th

1. ***Review strain list on page 3 and check the blackboard for additional instructions.*** Transfer 2 L3 or 2 L4 ***wild-type*** **hermaphrodites** each to HT115(DE3)/pL440(*bli-1*), HT115(DE3)/pL440(*dpy-11*) and HT115(DE3) seeded plates. Incubate at 16-18°C until offspring reach adult phase, as phenotypes will be observed in adult offspring of these worms.
2. For each ***C. elegans*** **reference strain**, transfer 2 L3 or 2 L4 hermaphrodites to regular worm plates.
3. Incubate at 16-18°C until offspring reach adult phase, as phenotypes will be observed in adult worms.

Tuesday Dec 4th

1. Observe phenotypes of worms on each of the RNAi "feeding" plates and compare to the phenotypes of the reference strains
2. Determine ***penetrance*** and ***expressivity*** for the mutant and RNAi knockdown phenotypes according to the detailed instructions below.

Penetrance and Expressivity

Penetrance describes the proportion of individuals with a particular genotype that express an associated phenotype.
Expressivity refers to variations of a phenotype in individuals carrying a particular genotype.

RNAi changes the expression of the gene, rather than the genotype. Geneticists have expanded the concepts of penetrance and expressivity to include the ability of dsRNA to alter phenotype. That is, it is useful to determine the percentage of subjects treated with dsRNA that display the phenotype (penetrance), and the variation observed (expressivity).

Penetrance

1. Determine the penetrance of mutant and RNAi treated plates. Remember, penetrance only considers whether individuals express the trait or not. Thus, this result will be expressed as a percentage.
2. Observe at least 25 worms on each, or as many as are on the plate, if <25 are present. Record your results in your notebook, and on the class "overhead" sheet.

Expressivity Expressivity can be qualitatively or quantitatively characterized. We will use a "semi-quantitative" method. We will use a point measurement system. You will select the characteristics to "measure"…length, width, number of blisters, etc.
3 – strong
2 – average
1 – super weak/absent

1. Observe the mutant phenotypes. Is there a range of expression? If you see a range, set the "average" to a value of three. If there is no range then all subjects are valued "3".
2. Observe the RNAi phenotypes. Is there a range of expression? If you see a range, is it greater, or smaller than you observed in wt.
3. Set *your* scale…use the observation (mutant or RNAi) with the widest range to set the limits of your scale.
4. Observe at least 25 worms on each, or as many as are on the plate, if <25 are present. Record your results in your notebook, and on the class "overhead" sheet.

Widen your thinking: mutant alleles that yield less than 100% penetrant results alter Mendelian ratios. Consider a recessive allele that is 80% penetrant when homozygous. What phenotypic ratio would you expect in a population an F2 population of 500 segregating for such an allele?