Caenorhabditis elegans: The Heavyweight Champ of Gene Knockout Technology

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The nematode (worm) *Caenorhabditis elegans* was the first multicellular organism to have its genome, or complete DNA sequence, sequenced by human beings. The sequencing project for *C. elegans* was completed in 1998[1] and entailed the examination and processing of 19,000 genes, or a total of 97 Mb of data . The task of sequencing the complete genome of an organism is a daunting challenge, one only surpassed by the still greater project of determining the function of each gene. But through the use of reverse genetics on the genome sequence of *C. elegans*, researchers can systematically determine what the function of each gene is. Reverse genetics involves deducing how a gene works by examining its DNA sequence and deducing the function, or lack thereof, of its mutant counterpart.

Eventually biologists will determine the function of each gene in *C. elegans*. Since many organisms contain similar genes with similar functions, this information can be applied to other organisms which will help make the functional analysis of other genomes easier and faster.

To take on the overwhelming task of the functional analysis of the *C. elegans* genome, the "Gene Knockout Consortium" was created[2]. It is currently an international collaboration between three labs whose mandate is to "produce null alleles of all known genes in the *C. elegans* genome"[2], which means they plan to find these null alleles by "knocking out" (rendering inactive) a gene of interest in an attempt to determine its function. The three labs involved in the *C. elegans* Gene Knockout Consortium include:

- Don Moerman's lab at the University of British Columbia
- Robert Barstead's lab from the Oklahoma Medical Research Foundation
- Shohei Mitani's lab at the Tokyo Women's Medical University School of Medicine

The consortium welcomes any submissions on *C. elegans* knockout genes. They provide the appropriate strain of *C. elegans* to other labs that may want to participate. By doing this, the consortium is offering other labs both the opportunity and the materials for further research , while gathering a list of all the knockout genes being created.

Knockout genes are traditionally obtained using a technique called chemical mutagenesis, where mutants are created by exposing the gene to chemicals or UV light [2]. Nested PCR (polymerase chain reaction) methods are then used to determine whether a deletion has occurred in the desired region. If it has, then an effective knockout has been created. The worm will then be taken for further study. This method, used in two of the consortium labs, has been a very popular technique. But recently, a new method called RNA interference (RNAi) has become even more widespread among molecular biologists. RNAi is done by injecting double stranded RNA with the desired knockout sequence into the worm genome[3]. This produces a dysfunctional mutant worm since the injected RNA binds to its normal, homologous mRNA while it is exiting from the nucleus, rendering it inactive, which in turn stops protein synthesis intiated by the original, functional gene. There are advantages and disadvantages to both methods. Currently the RNAi method is growing in popularity.

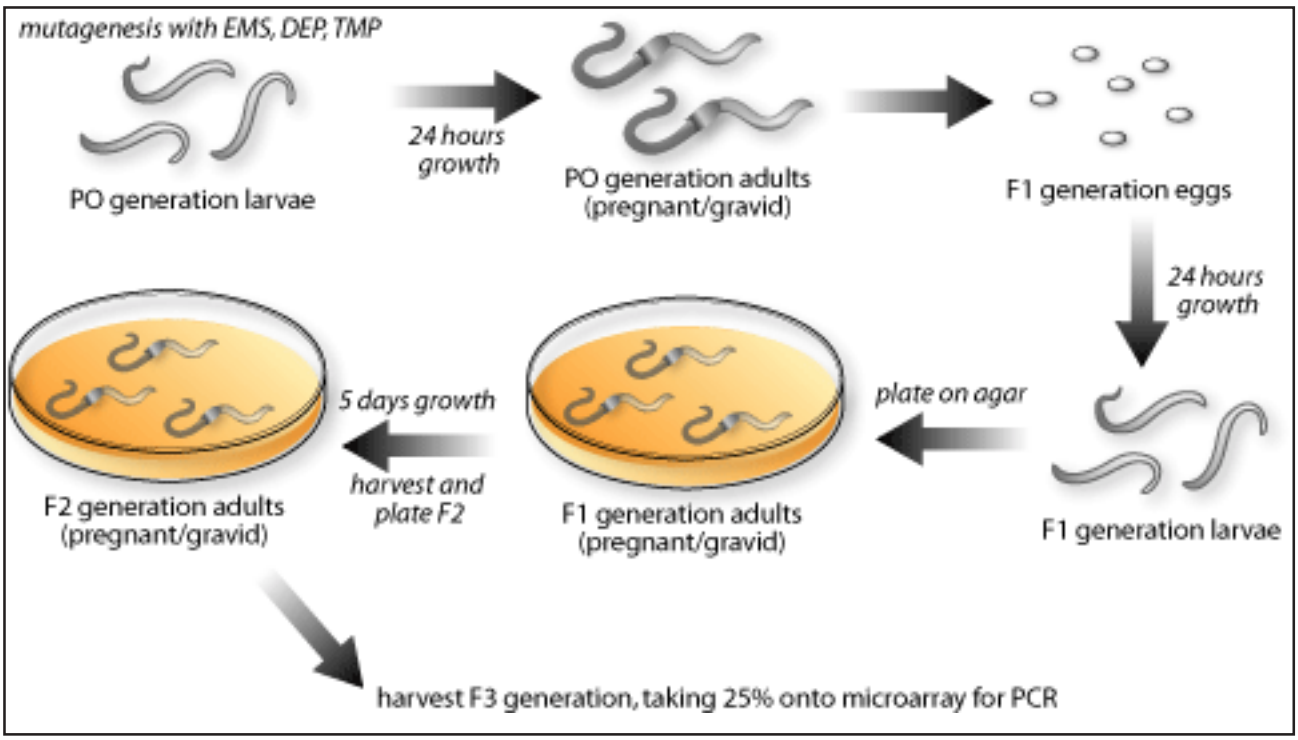

Figure 1. Chemical Mutagenesis of Caenorhabditis elegans.

Chemical Mutagenesis

Chemical Mutagenesis of *C. elegans* is obtained by first treating the worms with either EMS (ethyl methane-sulphonate) or TMP/UV (trimethylpsoralen with UV irradiation). EMS has an average mutation frequency of $5x10^{-4}$ mutations per gene, or a chance of one in 10 000 genes becoming mutated. 13% of those mutations are deletions. TMP/UV has an average mutation frequency of 3×10^{-5} mutations per gene, or odds of one in 33 333. 50% of the mutations are deletions[4].

The worms that are treated are usually hermaphrodites at the adult or young adult stage. After they have been mutagenized, the worms will be cultured in a plate with agar and bacteria as a food source. These mutagenized worms (also called the P0 generation) are left on the plate to lay eggs (each will usually lay approximately 75eggs). They are then washed away, and their eggs are harvested to be grown into F1 worms. The F1 worms are then further plated at 500 worms a plate. These plates are harvested again and the eggs are once again plated to yield F2 worms. The F2 worms are allowed to lay eggs and once again the plates are harvested to yield F3 worms. 25% of the adult F3 worms from each plate are then loaded onto an array for PCR. The other 75% of the worms are kept in an incubator at low temperature to slow their growth[5].

To ensure that mutagenesis has occurred, a screen may be set up to spot for known mutant phenotypes in P0. For example, it is known that deletion of the unc-22 gene will produce a worm with the phenotype of twitching. Since unc-22 is a large gene, the chance that mutagenesis will occur in that gene is high. If after screening, no twitching phenotypes were observed then mutagenesis may not have been successful. Moerman's lab advises that at least half of their observed plates contain this phenotype before they go forward with harvesting the worms[6].

This procedure may vary from lab to lab but the general outline is the same. The worms are cultured up to the F3 generation before they are used for PCR. Some labs may freeze the plates of unused F3 worms instead of incubating them. Unless there is great confidence that the recovery of frozen worms will be successful, this is usually not done[4].

In order to determine whether a deletion in a particular gene has occurred in the worm, nested PCR is performed. The primers for the PCR should flank the region of the target gene. Multiple sets of primers

may be used at the same time for different target genes. PCR is then performed on each well of the array. The PCR product is then run on a gel to determine if a deletion has occurred. A DNA band that weighs less than the target gene will indicate that a deletion has occurred[4].

After locating which well of the array has a deletion, the corresponding plate of worms can be harvested and the F3plate of 500 worms can now be divided into 100 plates of 5 worms. Each plate can then be put through PCR again to pinpoint which worm has the deletion. This is done until the deletion can be attributed to one worm[5].

After the worm with the particular deletion is identified, researchers may want to sequence the deleted gene to determine which region was deleted. The mutant phenotype for the deletion will be recorded. Many *C. elegans* knockout sequences have been obtained through this method.

RNAi

RNA interference (RNAi) is a method that was discovered a few years ago that can inhibit a particular gene's function by effectively stopping protein synthesis at the mRNA level. The double stranded RNA (dsRNA) used to bind to the worm's mRNA should match the sequence of the gene for which you want to inhibit function. dsRNA is delivered into the worm either by injection, feeding or soaking. dsRNA can be synthesized from a cDNA library. The presence of dsRNA dramatically decreases the amount of corresponding mRNA being phenotypically expressed[5].

Only a small amount of dsRNA is required for it to work. It was found that a dilution of about two molecules of dsRNA per cell still induced interference[3]. This suggests that there may be a catalytic effect at work since two molecules per cell intuitively seems far too low to directly interfere with cell activity. It is known that RNAi will only work if dsRNA codes for exons, which are sequences of DNA that code for protein synthesis. If dsRNA coded for introns (which do not code for proteins) then the mutant phenotype will not appear[3]. This indicates that RNAi works at a posttranscriptional level. The effects of dsRNA will also appear in the next generation. This may be due to the presence of dsRNA in germline cells or reduced maternal mRNA in the embryo[7]. The mechanism of precisely how RNAi works is still unclear.

RNAi was used recently in several large-scale analysis efforts of *C. elegans* knockouts. RNAi was also used in the analysis of chromosomes in *C. elegans*[8]. This was accomplished by using dsRNA on all the known genes in a chromosome. For example, Sugimoto used 2479 genes from cDNA clones and prepared dsRNA from each gene[9]. Worms were then soaked in the dsRNA solution for a particular gene. They then logged the phenotype of all the worms and found that 675 genes show a visible mutant phenotype. Other groups followed similar procedures but narrowed their focus to a specific chromosome. By doing so it is possible to determine how many genes in the whole *C. elegans* genome will display a mutated phenotype after RNAi treatment.

RNAi can also reveal valuable information about the chromosomal arrangement of genes. For example, from Sugimoto's paper it was found that on the X chromosome of the worm only 16.4% of the genes showed mutations when RNA interference occurs[9]. This suggests that the X chromosome may have an unusually high number of introns, since there is such a low phenotypic expression of mutation. An evolutionary explanation proposed by Maeda is that if the X chromosome held many essential genes then *C. elegans* males, which only possess one sex chromosome (XO), would be especially vulnerable to lethal genetic dysfunctions since they do not possess an extra X chromosome as a back up, as do hermaphroditic *C. elegans* worms.

RNAi can also be used to discover the existence and function of suppressor genes. By using a worm with both a genetic mutation and a normal phenotype, a suppressor gene for the mutated gene can be determined by subjecting the mutated worm to RNAi. Any worm that subsequently exhibits a mutant phenotype after treatment may be demonstrating that a defect in a suppressor gene is in effect. Although this may take a longer time to perform, new automation RNAi techniques will eventually make this process easier.

Chemical mutagenesis vs. RNAi

The introduction of RNAi allowed scientists to analyse post-genomic sequencing data quickly and easily, relative to traditional chemical mutagenesis. Preparation for RNAi is also much simpler and less time-consuming than the preparation for chemical mutagenesis. Although it may be easier for large-scale analysis and for performing knockouts, there are a few key features of RNAi that are disadvantageous to the researcher, depending on the purpose of the experiment.

RNAi inhibits gene functions at the posttranscriptional level while chemical mutagenesis inhibits gene functions at the genomic level. Because of this, RNAi has the ability to inhibit mRNA that the worm has taken from its mother. This does not happen in chemical mutagenesis because even though the gene itself is disabled, there may still be residual maternal mRNA in the embryo or offspring that will enable the organism to function normally. Therefore, it is not surprising if RNAi sometimes produces a more severe mutant phenotype than chemical mutagenesis.

Chemical mutagenesis is somewhat flawed because it is much more difficult to determine if a gene has a lethal mutation. It is harder to detect the presence of a lethal mutation because there may not be enough worms on which to perform PCR, since the mutant frequency for any mutagenesis technique is quite low. Repeated attempts to get the same lethal mutants is very difficult since mutation is random. It is much easier in RNAi to determine which gene is causing lethal mutations. It is also possible to combine RNAi and chemical mutagenesis together to determine whether maternal mRNA is reducing the worm's disability, or even averting its death. For example, if a worm exhibits a normal phenotype after creating a deletion in a gene using chemical mutagenesis, even though it has a lethal phenotype when the same gene is interfered by RNAi, this may indicate that maternal mRNA may be rescuing the worm.

Specificity is very important in reverse genetics since you are starting work from the genotypic to the phenotypic level. RNAi allows researchers the luxury of picking which gene they want to inhibit and it produces quick results. Since chemical mutagenesis is completely random, extensive screening procedures must be in place to pick out the desired mutants.

With RNAi, it is easier to carry out categorical analysis of genes because of its specificity. For example it is much faster to put worms through an RNAi analysis and then count the different kinds of phenotypes. And since the gene that is being inhibited is always known, it is easier to make a list of a certain types of mutants (i.e. a list of germline mutants).

One of the shortfalls of RNAi is that it is possible to 'cross-interfere' with similar and related genes[7]. Thus producing phenotypic mutations that may not be due solely to the interference of one particular gene. This can now be avoided because the genome has been fully sequenced. It is possible to make dsRNA using only unique RNA regions of the gene. It is also possible for RNAi to focus on specific tissues. This is because RNAi cannot spread to other parts of the body. While it can enter other cells, there are certain limits on how far RNAi can spread. It is also possible for some cell types to express resistance in RNAi [7].

Chemical mutagenesis still has distinct advantages over RNAi because it mutates at the genomic level, therefore gene interactions can only be studied with chemical mutagenesis. RNAi is able to simplify functional analysis of *C. elegans*. It is faster and more precise. With the possible automation of this method in the future, large-scale analysis will become much less time-consuming.

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