

The mechanism RNA interference

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Hypothesis and Specific Aims

Uptake of double-stranded RNA (dsRNA) into some types of eukaryotic cells results in nearly total depletion of homologous cellular messenger RNA, in a process termed RNA interference (RNAi). This specific loss of message is both dramatic and long-lived; days after uptake of dsRNA, only slight amounts of homologous mRNA can be found in the nucleus, while the cytoplasm is devoid of homologous message. dsRNA is essential for this effect, as uptake of single stranded RNA (ssRNA) gives variable results (at times sense RNA results in depletion, but the antisense does not, and vice versa). This effect does not arise from alterations in initiation of transcription, splicing or changes in the DNA sequence. Furthermore, RNAi is highly specific. Only messages complementary to the dsRNA are degraded, as the levels of messages slightly less homologous are not altered. This effect is also catalytic: only sub-stoichiometric amounts of dsRNA relative to homologous mRNA are needed per cell to confer nearly total homologous mRNA depletion. Moreover, the RNAi effect can cross cell boundaries to affect progeny. In addition to its potential use as a possible pharmaceutical method for disease management, understanding of the mechanism of dsRNA mediated homologous mRNA depletion may provide insight into mechanisms of cellular defense of viral dsRNA infections as well as cellular housekeeping of mRNA levels.

I propose the following hypothesis: the mechanism of dsRNA mediated homologous mRNA degradation requires specific structural components of the dsRNA in addition to cellular factors to create a catalytic element that results in permanent, specific cellular mRNA depletion. To test this hypothesis, I am proposing two specific aims:

Specific Aim 1: Determine the minimal length, homology and structural requirements necessary for RNAi. dsRNA constructs will be created from the *C. elegans*' genes *unc-22*, *unc-54* and *fem-1*. These messages have been used successfully for RNAi. The dsRNA constructs that will be created from these genes will have altered length, homology and structural requirements. These constructs will be introduced into *C. elegans* to elucidate the structural requirements necessary for RNAi.

Specific Aim 2: Determine that RNAi is a permanent effect rather than a temporary effect. dsRNA will be introduced into dauer *C. elegans*. In the dauer alternative development state, *C. elegans* can live up to eight times longer than the two to three week lifespan of the non-dauer worm. The extended lifespan of these animals will facilitate the assessment of the time dependence of the RNAi effect.

Background and Significance

The goal of genetics is to understand how genetic information specifies development, anatomy and behavior of a living system. To realize this goal, tools have been developed to bridge the gap between sequence information and functional information. One of these techniques is termed RNA interference (RNAi). This technique evolved from the theory that injections of antisense RNA into *C. elegans* would deplete the nematode's cells of homologous sense mRNA by binding to the mRNA and preventing translation. Therefore, it was hypothesized that this technique would allow researchers to phenocopy a null mutation in *C. elegans* by depleting the pool of sense mRNA. After injecting the antisense RNA, a phenotype consistent for a knockout for the corresponding gene was observed. However, the injection of sense RNA yielded the same phenotype (1). Since these initial experiments, the effectiveness of the technique to create knockout mutants (by some unknown mechanism) but the apparent lack of strand specificity has been demonstrated by other researchers (1, 2). The mystery surrounding the mechanism by which exogenous RNA confers a phenotype in *C. elegans* deepened when it was observed that double-stranded RNA (dsRNA) introduced into *C. elegans* was at least an order of magnitude more potent in conferring a phenotype than single-stranded antisense or sense RNA alone (3). Since that time, Fire and colleagues have theorized that the single-stranded RNA used in previous studies may have been contaminated by dsRNA, and this dsRNA was the substance that conferred the observed phenotype (3). The introduction of dsRNA into an organism was then given the name RNAi.

Since its first introduction, the technique of RNAi (injecting or otherwise introducing (see below) dsRNA into a living system) has gained wide acceptance as a genetic tool and is used commonly in *C. elegans* (4, 5, 6). In addition, it has been demonstrated in both *Trypanosoma brucei* and *Drosophila melanogaster* (7, 8, 9). Observations made after introduction of dsRNA into these animals have resulted in a growing body of evidence that supports the theory that RNAi has uncovered a normal function of cellular metabolism -- the degradation of homologous mRNA after challenge with dsRNA. The idea that the cell may be challenged with dsRNA in nature is not new. For example, the hypothesis that dsRNA may result in gene specific silencing was proposed in 1997 by Wutz et al. Their findings suggest that the activity of an antisense promoter in the first intron of the gene for the mouse receptor for the insulin-like growth factor type-2 (Igf2r) is important for its paternal-specific repression (10). In addition, dsRNA has also been recognized as a potent signaling molecule in the induction of interferons and the execution of the antiviral state (11). Finally, Sharp in his 1999 Genes and Development article argues that "restriction of infection by RNA viruses is almost certainly one of the biological consequences of the...RNAi state."

There are many remarkable characteristics of this putative RNAi pathway that will be collectively referred to as the 'RNAi effect.' For clarity, I will focus on the effects observed in the *C. elegans* system. First, and perhaps the most dramatic of all the RNAi effects is the total depletion of homologous, cytoplasmic mRNA from the organism's cells, even at very early time

points. *In situ* hybridization studies and studies involving GFP-expressing worms have failed to detect homologous mRNA messages in the *C. elegans* embryo after introduction of homologous dsRNA into the germline cell (12). Secondly, this effect is seen when an estimated one or two molecules of dsRNA are taken up by the cell, indicating that this effect has a catalytic component. Thirdly, the depletion of cytosolic homologous mRNA can be transported across cell boundaries. For example, when the dsRNA is introduced into the gut of the worm either by injection or ingestion of bacteria expressing dsRNA, surrounding tissues will be depleted of the homologous mRNA. It has also been demonstrated that soaking worms in a solution of dsRNA creates the RNAi effect in the tissue cells (13, 14). In addition to crossing tissue cell boundaries, the dsRNA challenged worm's progeny display the RNAi phenotype, presumably resulting from dsRNA entering the germ cells. Finally, the RNAi effect appears to be long-lived, however its persistence over time has not been documented. A potent RNAi effect can not only be found in the injected animal for at least the time it takes the animal to reproduce, but also in the injected animal's progeny for an undetermined amount of time (3).

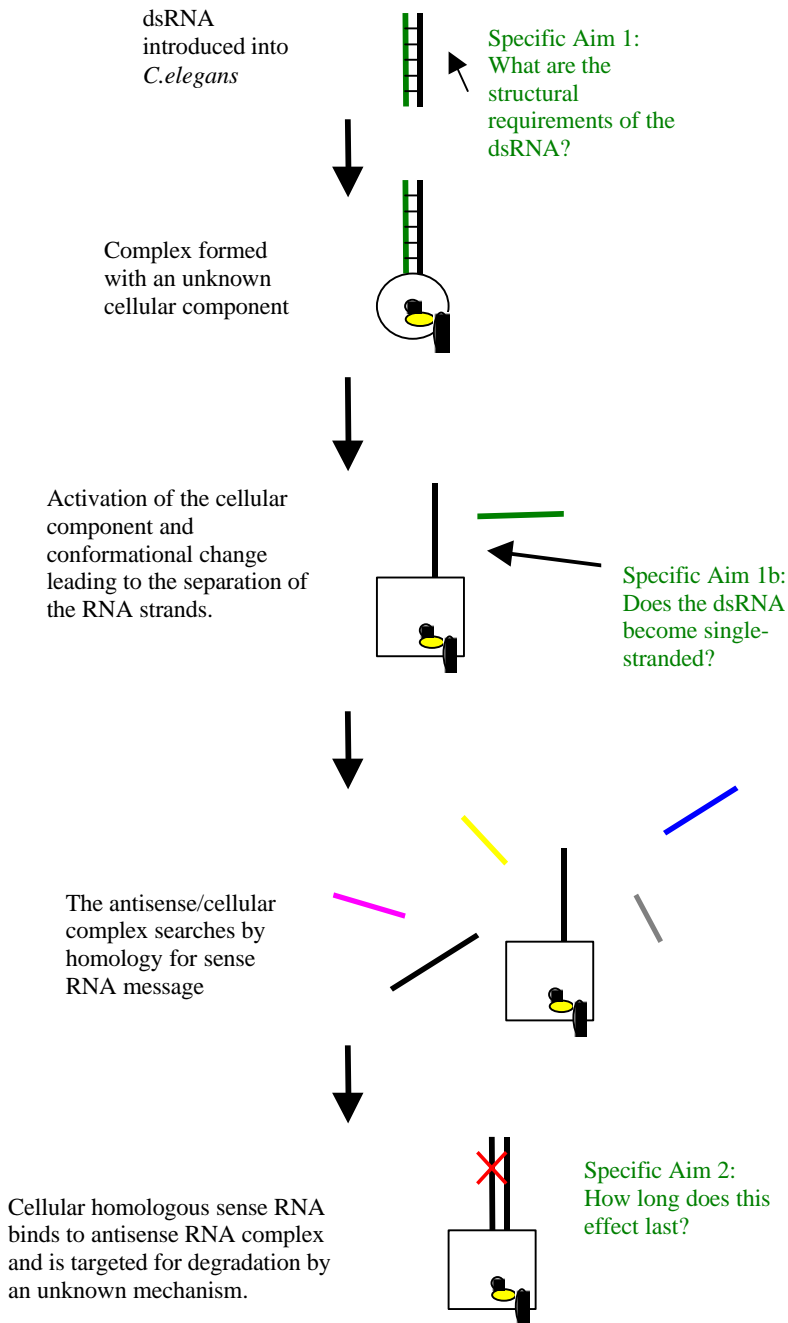
The fact that dsRNA can cross the germ cell and tissue cell boundaries of soaked worms and worms ingesting dsRNA expressing bacteria, coupled with the knowledge that *C. elegans* can encounter double-stranded RNA in its environment (10, 11), suggests that the mechanism of RNAi is not a byproduct created by injection of dsRNA into an organism. These data suggest that the injection of dsRNA has tapped into a mechanism by which the cell degrades targeted homologous mRNA as a "normal" function of the cell. Although some studies have been performed looking at the mechanism of RNAi, there have been no systematic studies. If this mechanism is elucidated, many avenues for further research, genetic manipulation and therapeutic techniques may emerge. Therefore, in order to fully characterize this effect, it is important to examine what is known about the mechanism of RNA interference and what is currently lacking.

To date, it is known that the RNAi effect does not act at the DNA sequence level. No modifications in DNA sequence are observed in any of sequences analyzed from affected animals. Additional data for this are that for the vast majority of the of the different dsRNA constructs injected, the F1 generation displays the RNAi phenotype, but 100% of the F2 progeny reverts back to wild-type (3), indicating that there are no long-lasting heritable consequences. There are reports in the literature that in certain instances the RNAi effect can last over many generations (A. Grishook and C.C. Mellow, unpublished results). However, this is believed to be a result of DNA contamination in the dsRNA preparation (Fire, personal communication). This DNA may have inserted into the worm's genome, perpetuating the RNAi effect.

In addition to the data that RNAi does not change the DNA at the sequence level, data have also shown that the action of RNAi appears to be at the post-transcriptional level, as introns and promoter sequences are not sufficient to produce a phenotype. Further studies indicate that transcription is unaffected, revealing that endogenous mRNA targeted by the dsRNA transiently accumulates to some level in the nucleus before being depleted. Moreover, in *C. elegans* the

product of the *smg* (suppressor affecting *message stability*) gene (which has been identified as constituting a system that degrades translationally aberrant mRNAs and may play a role in normal mRNA degradation (15, 16, 17) is not required for RNAi operation. The lack of SMG requirement for effective RNAi suggests the involvement of other, uncharacterized RNA degradation mechanisms (3, 18).

The above data coupled with evidence that the injected dsRNA does not self-replicate has led to the following model shown pictorially below. During RNAi, the dsRNA interacts with a



hypothetical cellular component, perhaps a protein or riboprotein complex, that undergoes a conformational change. This change would not only activate the complex for RNA degradation, but also allows separation of the sense and antisense strands. This altered complex would then search for homologous cellular mRNA based upon the sequence of the antisense RNA. After the annealing of a cellular sense mRNA strand, the complex would somehow mark the sense RNA for degradation. Possible mechanisms for marking include direct cleavage of the target mRNA, covalent modification (e.g. by adenosine deaminase), or the recruitment or removal of specific RNA-binding proteins. The target mRNA would then be rapidly degraded by cellular mechanisms involved in removing damaged mRNA (3).

The model predicts many areas to test in order to elucidate the mechanism of RNAi. Specifically, what are the structural requirements needed for RNAi and how long does this effect last? Elucidation of these areas will reveal much about the mechanism of the RNAi effect.

Research Design

Uptake of exogenous dsRNA results in a dramatic decrease in homologous cellular mRNA. Our previous work in *Caenorhabditis elegans* indicates that only dsRNA homologous to the processed mRNA causes this effect. In addition, double stranded portions of the promoter and introns do not initiate mRNA depletion. This suggests that specific structural and homology requirements are needed to initiate the RNAi effect. We plan to test this hypothesis and elucidate the structural requirements needed for dsRNA mediated homologous mRNA depletion, also known as RNA interference (RNAi). We will use the term *RNAi effect* to describe the worm's phenotype observed after uptake of a particular dsRNA and the loss of targeted mRNA.

Aim 1a: A minimal length of dsRNA is required for RNAi. Due to the specificity of the mRNA depletion, we believe that a minimum length of dsRNA will be found for which shorter dsRNA molecules do not support RNAi. We plan to assess the dependence of RNAi upon the length of dsRNA molecules by targeting three genes in the nematode *C. elegans*, namely *unc-22*, *unc-54* and *fem-1*. We chose *C. elegans* as a model system because the anatomy and development are well characterized, sophisticated genetic techniques are available and the genome sequence has been recently published. We chose to use the exon portions from the genes *unc-22*, *unc-54* and *fem-1* to introduce RNAi because deletion of these mRNA products from *C. elegans* creates loss-of-function mutants with distinct phenotypes. The *unc-22* gene codes for an abundant but non-essential protein in *C. elegans* muscle cells. Deletion of this gene's mRNA results in defects in the muscle cells that leads to a twitching phenotype (3, 19, 20). The *unc-54* gene codes for a non-essential myofilament protein (21); deletion of this gene results in a paralysis phenotype. Deletion of the *fem-1* gene in *C. elegans* results in an all-female population (i.e. no hermaphrodites) (22, 23). These three phenotypes can be quickly and easily identified in individuals by light microscopy. In addition, *unc-22*, *unc-54* and *fem-1* gene products have been used successfully for dsRNA mediated homologous mRNA depletion (3, 18).

We will create dsRNA variants from an established procedure by transcribing from the available plasmid clones of *unc-22*, *unc-54* and *fem-1* using T3 and T7 polymerase (see Methods). Following synthesis of the RNA, the templates will be removed by Dnase treatment. After verification of the double-stranded nature of the transcribed RNA, we will introduce the dsRNA molecules into the nematode as described below and in reference (24). We will begin our studies using the same length of RNA that was used previously with success: 742 bases out of 22 kilobase mRNA for *unc-22*, 546 bases out of the eight kilobase mRNA for *unc-54* and 531 bases out of the approximately 2 kilobase mRNA for the *fem-1* gene. Three methods of RNA introduction are currently available. Our method of choice is injection of the dsRNA solution into one of the two gonad arms (see Methods), which has been shown previously to be sufficient for RNAi (3). Although this method is laborious, it is a standard and widely used technique in *C. elegans* manipulation. Injection of dsRNA material has the benefit of a very high success rate

with RNAi. All worms successfully injected show severely decreased or non-detectable levels of the targeted cellular mRNA, display a phenotype similar to a knock-out mutant for the corresponding gene produce F1 progeny that are nearly homogenous in their expression of the same phenotype. The other methods of RNA introduction include ingestion of bacteria expressing dsRNA and soaking of the nematode in a dsRNA solution. However, each of these methods has a lower success rate of 85% and 80%, respectively (13, 14).

For injection experiments, we are defining successful homogeneous mRNA depletion (RNAi effect) based on two criteria: (1) the nematodes display the phenotype characteristic for loss-of-function mutants associated with the gene introduced and (2) *in situ* hybridization shows no expression of homologous mRNA, while still expressing other mRNA closely related in sequence to the injected RNA. For each dsRNA construct made, initial experiments will consist of injection of the dsRNA solution into a minimum of ten nematodes. Nematodes typically have 200 progeny; however, due to differences in egg maturity, only about 150 progeny will be affected, resulting in approximately 1500 RNAi worms. Thirty affected nematodes for each construct will also be studied by *in situ* probing. Once it is found that a minimum length is needed for expression of RNAi, we will increase the number of nematodes injected and subsequently examined to explore this data.

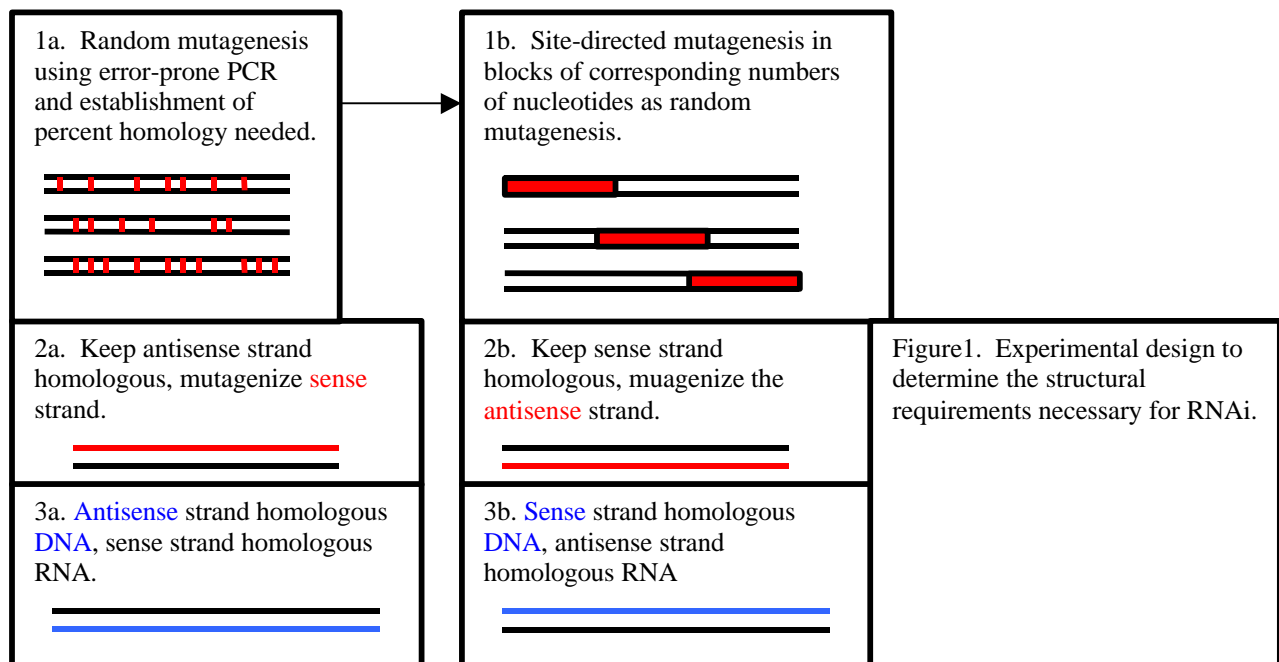
Aim 1b. Minimal homology between the RNA and cellular mRNA is required for

RNAi. As stated previously, due to the specificity of this effect, one can strongly suggest that a minimum amount of sequence homology is necessary between the dsRNA and the target gene for depletion. We will test the hypothesis that minimal homology is required for RNAi by assessing the phenotype of nematodes containing dsRNA with decreased homology to the target cellular mRNA. To test this theory, we will use the approaches outlined in Fig.1. Panel 1a. depicts the random mutagenesis of both strands of the dsRNA until there is no RNAi effect as determined by the absence of phenotypic expression and detectable levels of homologous cellular mRNA by *in situ* hybridization. Once the percent dissimilarity for the three target genes has been established, we will introduce block patches of dissimilarity with lengths corresponding to the minimum number random substitutions allowed in the dsRNA that does not ablate the RNAi effect (Fig. 1b). We will use these patches of dissimilarity to 'walk' across the dsRNA molecule to determine if particular areas of the dsRNA are more sensitive to mutations.

One possible outcome of these experiments is that each gene may require a different level of homology. If so, that would suggest that percent homology necessary for RNAi is determined on a gene-by-gene basis. Another possible outcome, which is not mutually exclusive of the first, is that mutations in certain regions of the double-stranded RNA effect RNAi more than others. This would suggest that the mechanism utilizes these segments to help in depletion of targeted cellular mRNA.

Panel 2a.-2b. depicts testing the homology requirements of RNAi by creating constructs in which one stand is not very homologous to the target mRNA, but the opposite strand is

homologous to the mRNA. The percent dissimilarity created in the mutagenized strand will be influenced by the data gathered in the experiments shown in 1a. and 1b., but is not dependent upon them. These constructs will be introduced into the worm. Then, the injected worm and its progeny will be examined for a decreased number of affected individuals by phenotypic assessment and *in situ* hybridization. If only one strand seems more sensitive than the other strand (for example antisense more sensitive than sense), it suggests that the mechanism of RNAi is dependent upon that strand. However, if mutations in either strand have the same affect, then it would seem that RNAi is not specific for either strand. If neither strand has an effect, then we will continue to mutagenize one strand or the other until we can resolve some type of difference, such as decreased duration of mRNA depletion (Specific Aim 2), lack of mRNA depletion and/or non-expression of phenotype.



Panel 3a.-3b. depicts the third strategy we will utilize to elucidate the homology required for the mechanism of RNAi. We plan to anneal two complementary strands of nucleic acids, a DNA strand and a RNA strand (see Methods). The RNA strand will correspond to the non-mutagenized RNA strands used in the above experiments, while the DNA strand will be complementary to the RNA strand. After injecting the RNA/DNA hybrid into the worm, we will assess the worms for RNAi by looking for phenotypic expression and depletion of target mRNA. No deviations from duration of the RNAi effect, or number of worms affected would indicate that the mechanism is tolerant of the DNA strand. If, on the other hand, the DNA strand affected the mechanism we can conclude that the mechanism was sensitive to the structural differences imposed by the DNA strand.

Aim1c. The dsRNA strands must disassociate to confer the RNAi effect. According to the model, the dsRNA must separate in order to bestow the RNAi effect. By utilizing cross-linked RNA for RNAi, we will determine that the dsRNA must become single-stranded. We will introduce into the *unc-22*, *unc-54*, and *fem-1* constructs cross-links with site-specific photoaffinity cross-links using azidophenacyl (APA--see Methods). Cross-links will be placed at the either or both ends of the construct as described in the Methods section. An outline of the scheme is presented in figure 2.

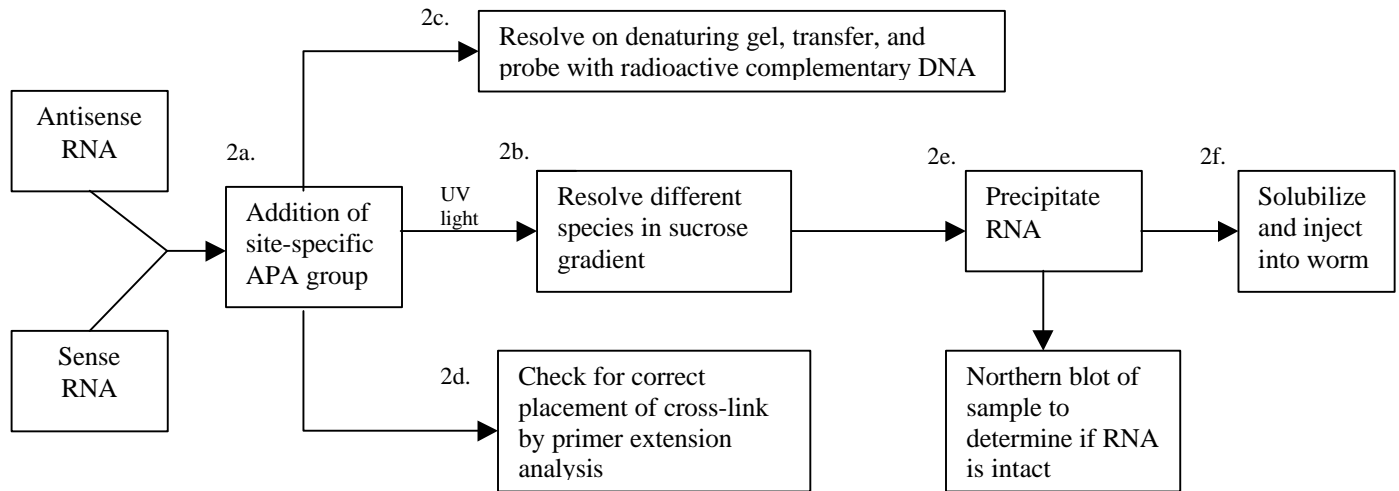


Figure 2. Design of experimental methods used to establish that the dsRNA must disassociate for the RNAi effect to be presented.

We will take the single-stranded RNA and add a site-specific APA group on one of the strands. After incubation of the antisense and sense strands together in annealing conditions, the strands will be subjected to 302-nm light to induce intermolecular cross-linking. Once it is established that the single-stranded RNA remains intact after the addition of the APA group and that the cross-links are in the desired positions for the dsRNA (see Methods), we will inject the purified, cross-linked dsRNA into the nematode (Methods). We predict that the RNAi effect will be sensitive to the placement of interstrand cross-links. Our model indicates that the RNAi effect will not be established if the sense RNA does not disassociate. This specific aim will directly test this hypothesis. Cross-links on either end or both ends of the dsRNA molecule will indicate if the mechanism of RNAi will continue to function with part of the strands stuck together. If this is the case, these experiments will also indicate which area of the dsRNA is must separate for RNAi to occur. If one interstrand cross-link is unsuccessful in inhibiting the RNAi effect, we will introduce more cross-links between the RNA strands to determine if more crosslinking has an effect. If no effect is seen with the cross-linking experiment, it may be because the strands do not separate and instead form a triple helix with the cellular mRNA. We plan to test for this by using both RNA/DNA hybrid molecules and dsRNA molecules that are mutagenized on only one strand (Aim 1b, Figure 1). An abolishment of the RNAi effect using these constructs may

indicate that either the antisense strand or the sense strand vital to the mechanism of the RNAi effect.

Aim2. RNAi is a permanent rather than transient effect. To test the hypothesis that the RNAi effect is a permanent rather than transient effect, we will encourage larvae to display the RNAi phenotype by injecting dsRNA into the parent and then induce the larval offspring into a dauer state (see Methods). The dauer larvae, which live up to eight times longer than a wild-type nematode (approximately 122 days versus 14 days) (25). This will allow a significantly increased amount of time to study the duration of the RNAi effect. Dauer larvae are an alternative L3 larvae and are arrested in this stage until environmental conditions improve; they then progress into L4 larvae and complete their normal life cycle. Among other changes, these larvae have decreased metabolism and transcription levels (25). Despite these changes, we feel that dauer larvae will make good candidates for our studies. We will control for differences between the non-dauer worms and the dauer worms by comparing the phenotype and mRNA depletion of the worms in parallel for the life span of the non-dauer worms. Our scheme for testing the temporal hypothesis involves injecting fifteen adult, hermaphroditic worms with a

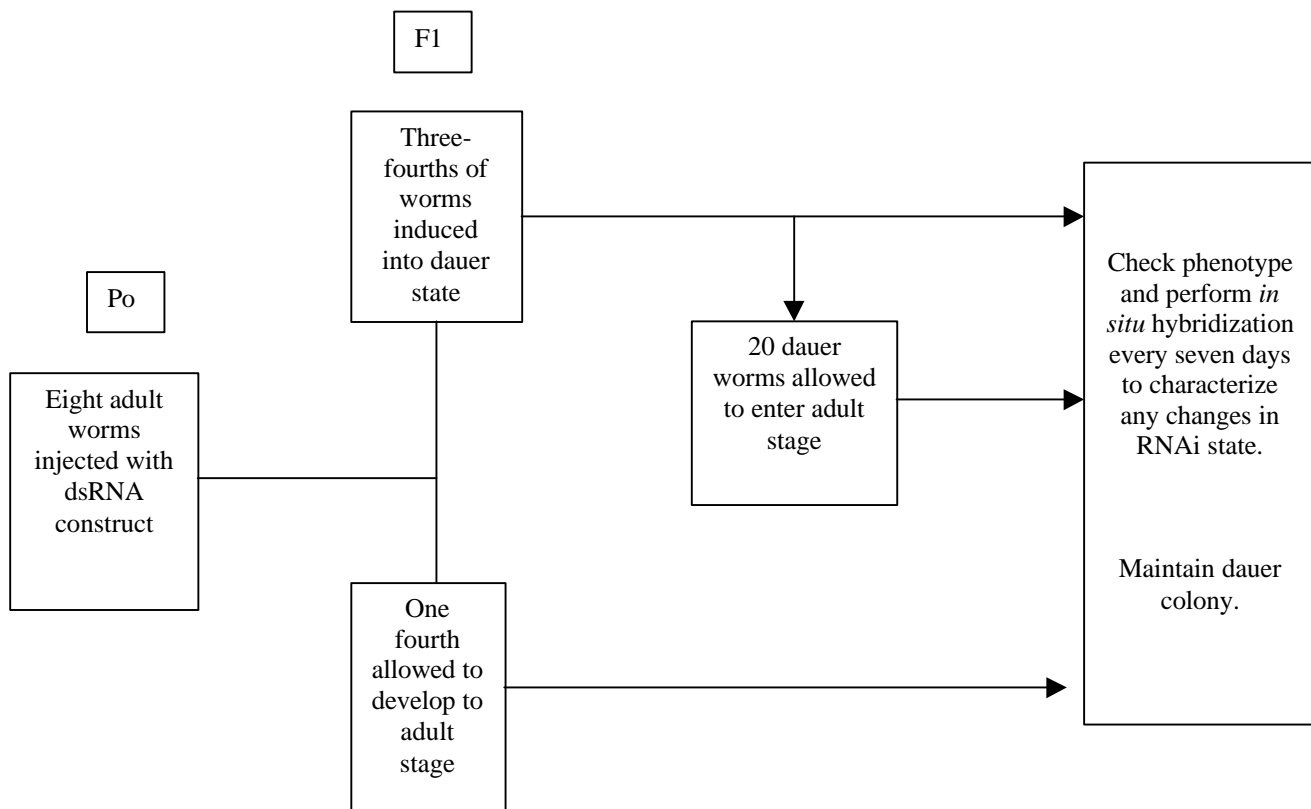


Figure 3: Schematic of the experimental design to assess the time dependence of the RNAi effect.

double-stranded RNA construct (Figure 3). After allowing them to recover and produce offspring, three fourths of those L1 larvae that display the knockout phenotype for the target mRNA will be collected and induced to the dauer state (see Methods). The dauer larvae will be separated from non-dauer worms (see Methods). The remaining one fourth of L1 larvae will be allowed to enter the adult state and used as controls. Every seven days the phenotype of all worms will be observed and 30 of them will be subjected to *in situ* hybridization: 10 from dauer state, 10 from dauer induced to adult, ten from adult injected population. After the adult worm population has expired, we will continue to maintain the dauer colony. Experiments will continue every seven days by inducing approximately 15 dauer larvae to enter the adult stage, observing the phenotype and checking for decreased targeted mRNA levels. This experiment will initially be performed with the *unc-22*, *unc54*, and *fem-1* constructs used in Specific Aim 1a. Then, for every construct that we use in Specific Aim 1 that gives us a RNAi phenotype, we will use in this experiment also. We expect that RNAi is not temporal; that it is an on or off event. Therefore we anticipate that the phenotypic expression and the absence of homologous mRNA will be typical of the dauer and adult affected worms at all time points. If, however, there are differences at different time points, those results will be vigorously investigated.

Methods

Creation of double-stranded RNA

DNA plasmid constructs (in our possession) of *unc-22*, *unc-54*, and *fem-1* serve as templates and are linearized with the appropriate restriction enzymes. The DNA is phenol/chloroform (1:1) extracted, precipitated in ethanol and resuspended in 5 mM Tris-HCl, pH 7.9 (TE). To create the RNA strands, (Rnase-free conditions), 1 μ g of resuspended DNA (4 μ l) is incubated in the following for 1.5 hours: 4 μ l TSC buffer obtained from Promega, 1 μ l each of 100 mM rATP, rGTP, rCTP, and rTTP, 36 μ l of Rnase-free TE, 1 μ l Rnasin, 2 μ l 100 mM DTT, 4 μ l H₂O, and 1 μ l of either T3 or T7 polymerase. The T3 reaction mixture is incubated at 25°C; the T7 reaction mixture is incubated at 37°C.

After the synthesis of the RNA strands, to ensure that the RNA strands are DNA-free, the above reaction mixture is incubated with 1 μ l of Rnase-free Dnase I for 15 minutes at 37°C. The reaction is stopped by adding 380 μ l of stop solution (1 M ammonium acetate, 10 mM EDTA, and 0.2% SDS) and 0.3 μ l of a 20 mg/ml solution of glycogen. The RNA is isolated in Rnase-free conditions by phenol/chloroform extraction and ethanol precipitation. The pellet is resuspended in Rnase-free TE and subjected to another round of Dnase I digestion using standard methods, incubated at 37°C for 1.5 hours and stopped with stop solution described above. The RNA is isolated by phenol/chloroform extraction and ethanol precipitation. Finally, the RNA is resuspended in 10 μ l of TE.

To make dsRNA for injection, equal volumes of sense, antisense and 3xIM (20 mM KPO₄ pH 7.5, 3mM Kcitrate pH 7.5, 2% PEG 6000) are mixed together. This mixture is incubated first at 68°C for 10 minutes and then at 37°C for 30 minutes. Finally, the annealing of the RNA strands will be verified by running an appropriate amount of RNA for each strand and the dsRNA reaction mixture on a standard TAE gel for visualization.

Microinjection of *C. elegans*

We will inject into one of the two gonad arms, as this is sufficient to produce the RNAi effect. The method of microinjection of *C. elegans* is described fully in (24), but is briefly outlined here. After coating a dry agarose injection pad with oil, individual worms are picked off of media plates with a worm pick and transferred to the pad for injection. Using a micromanipulator and a dissecting microscope, the needle loaded with the dsRNA solution is gently inserted into the apparent center of the gonad cytoplasm. Pressure is then applied to the needle, causing the RNA solution to infiltrate the gonad cytoplasm at the point of insertion. After injection, the worms are allowed to recover and are placed on appropriate media plates until further use.

In situ hybridization of *C. elegans*

Whole-mount in situ hybridization will be performed as described in (3, 26). Briefly, Adult worms are squashed to extrude the gonads and embryos prior to freeze cracking then fixed overnight in a commercially available fixative (Streck's tissue fixative-Streck Laboratories, Omaha, NE). To probe for mRNA, Digoxigenin (DIG)-labeled single-stranded DNA probes are synthesized by multiple copies of primer extension in the presence of DIG-dUTP using subclones generated from the plasmid constructs used in Specific Aim 1. An anti-DIG antibody conjugated to alkaline phosphatase is used to visualize the probes. All DIG components are available through Boehringer Manneheim.

Site-directed mutagenesis

Site directed mutagenesis will be carried out using Stratagene's Quick Change site-directed mutagenesis kit and protocol described therein.

Random mutagenesis

Random mutagenesis will be created in the RNA strands by first liberating the DNA template (to be used to synthesize RNA) from the plasmid. Next, randomly mutagenizing the DNA template using standard error-prone PCR techniques. Then, ligating the altered template back into the plasmid. These plasmids will be introduced into a bacterial host to create a random library of plasmids. Random colonies will be picked to liberate the DNA template and synthesize dsRNA (see Creation of double-stranded RNA). Percent mutation of the DNA or RNA strands will be determined by sequencing isolated plasmids from single colonies.

Creation of RNA/DNA hybrids

Annealing of RNA and DNA will be done according to the method of Palaniappan et al. (27). This method is outlined here. The RNA and DNA strands are annealed in 10mM Tris-HCl (pH 8.0), 1mM EDTA, and 80mM KCl. The mixed components are heated to 60 C for 10 minutes and cooled slowly for 90 minutes. Unannealed strands are removed by gel filtration spin chromatography. Th hybrids are analyzed by either native polyacrylamide or agarose gel electrophoresis to ensure the strands annealed at one to one ratios. Rnase H will be used when required to establish the presence of RNA/DNA hybrids.

APA crosslinking and analysis

For a full description of RNA crosslinking, see reference (28). Briefly, for 5'-modification, an azidophenacyl group is conjugated to the unique sulfur at the 5'-terminal phosphate of 5'guanosine monophosphorothioate (GMPS)-containing RNA strand. These RNAs are generated by *in vitro* transcription (as described previously) in the presence of GMPS. 3'-modification is accomplished by oxidation of the 3'-end of an appropriate RNA strand. Then, the primary amine-specific photoagent is attached by reductive alkylation with ethylene diamine. For crosslinking, appropriate single-stranded RNAs are annealed as described previously (one containing the photoaffinity crosslinking agent, one without) and irradiated with 302-nm light to convert the azido group to a highly reactive nitrene that is able to insert into a variety of covalent bonds. Controls and verification of crosslinking sites will be carried out as described in reference (28).

Primer extension analysis

Primer extension analysis, to determine the sites of crosslinking, will be carried out by the method of Harris, et al. (28).

Induction of *C. elegans* larvae into dauer state

L2 *C. elegans* larvae will be induced into the dauer state by pheromone stimulation as described in detail in reference (29).

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