GENE SILENCING BY DOUBLE STRANDED RNA

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by

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I would like to thank the Nobel Assembly of the Karolinska Institutet for the opportunity to describe some recent work on RNA-triggered gene silencing. First a few disclaimers, however. Telling the full story of gene silencing would be a mammoth enterprise that would take me many years to write and would take you well into the night to read. So we'll need to abbreviate the story more than a little. Second (and as you will see) we are only in the dawn of our knowledge; so consider the following to be primer... the best we could do as of December 8th, 2006. And third, please understand that the story that I am telling represents the work of several generations of biologists, chemists, and many shades in between. I'm pleased and proud that work from my laboratory has contributed to the field, and that this has led to my being chosen as one of the messengers to relay the story in this forum. At the same time, I hope that there will be no confusion of equating our modest contributions with those of the much grander RNAi enterprise.

DOUBLE STRANDED RNA AS A BIOLOGICAL ALARM SIGNAL

These disclaimers in hand, the story can now start with a biography of the first main character. Double stranded RNA is probably as old (or almost as old) as life on earth. Scientific recognition of this form of RNA is, however, a bit more recent, dating from the mid 1950s. The same kinds of base pairs of that can zip strands of DNA into a helix [1] were recognized just a few years later as being a feature of RNA structure [2–5]. When two RNA strands have extended regions of complementary sequence they can zip together to form a somewhat flexible rod-like structure similar in character (but distinct in detail [5,6]) from that of the DNA double helix.

The occurrence of double stranded RNAs in biological systems was uncovered in a number of experiments in the early 1960s [7–9]. Intriguingly all of the biological systems initially found to be sources for double stranded RNA involved virus infection. This data supported a proposal that many viruses might replicate from RNA to RNA through a double stranded RNA intermediate. At the time, the central dogma of molecular biology was being experimentally established, giving a clear indication that cells mainly used

double stranded *DNA* and single stranded *RNA* for long and short term information storage respectively. This left no place in normal cellular information flow for double stranded RNA, while leaving a key role (at least transiently) for dsRNA in replication of RNA viruses.

Our story next jumps back almost thirty years to a set of experiments that were directed toward an understanding host cell responses to viral infection [10,11]. These experiments involve two different (essentially unrelated) viruses infecting a single host (Figure 1). One virus was quite virulent and would kill its unfortunate host animal, while the second virus was relatively benign, causing only minor symptoms. The surprising result was that a preliminary infection with the benign virus could provide resistance to a subsequent challenge by the more virulent, nasty virus. The conclusion from these results is that the host (a rabbit in this case) has a way of knowing that it has been challenged by a viral pathogen and somehow sends itself a signal allowing resistance to further challenge. Although the ability of viruses to induce immune responses had been known for a long time, these results were unexpected by virtue of the apparent lack of relatedness between the two viruses used in the experiment. The generalized response to infection was

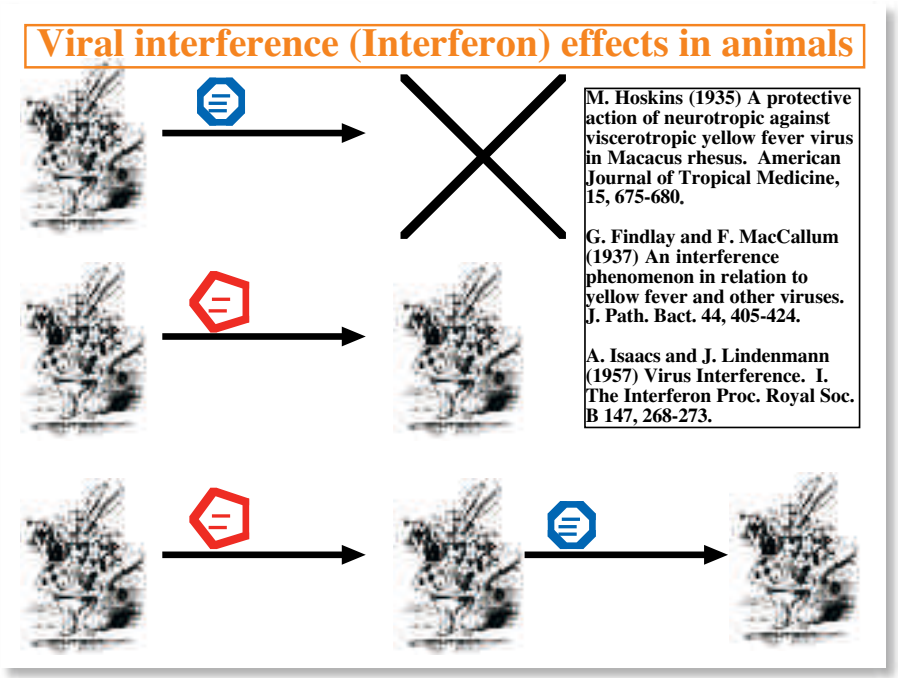

Figure 1. Diagram of viral interference effects ('innate immunity') in mammals. Top, A highly virulent virus (represented as a blue hexagon) will result in death if inoculated into a "naive" host animal. Middle, A less virulent and unrelated virus (represented as a red pentagon) infects cells but causes little or no systemic pathology, the animal remains alive. Bottom, a preliminary infection with the less virulent virus (red) leads to induction of an innate immune response which allows the animal to survive a subsequent challenge with the highly virulent (blue) virus.

a new phenomenon and led to an understanding of immune mechanisms that involve general alarm responses. A key follow-up to this observation was made about 20 years later when Isaacs and Lindeman [12] actually separated a protein component from the challenged animals that could transfer the general viral resistance when injected into naive animals. That protein component was called *interferon*.

In the course of this analysis, a physician/scientist named Richard Shope became interested in applying the innate immune response by finding treatments that would induce generalized immunity to provide viral resistance. Traveling the world at the end of the second world war, he collected biological materials looking for something that could be ground up and used as a starting material. His most notable success came from a fungus (*Penicillium funiculosum*) that he found in Guam growing on a picture of his wife Helen. Calling the extracts of the fungus "Helenine", Shope found that these could induce an interferon response in animals [13].

A next chapter in this early story was carried out by Maurice Hilleman's group at Merck, who used Shope's fungus as a starting point to purify the material that was actually responsible for the viral resistance. In a paper published in 1967 [14], they showed that double stranded RNA was present in the fungal extracts and was responsible for the induction of resistance. Given that there would have been little or no sequence similarity between the fungus-derived dsRNA and the viral target, they then tested additional very distantly related natural and synthetic double stranded RNAs and found that all could induce an interferon response [15–17]. There were (of course) many different questions raised by this study. Paramount perhaps was the question of why double stranded RNA was present in the fungus. Hilleman's publications suggested the intriguing hypothesis this was due to a fortuitous viral infection of the fungus. In fact, they had discovered an ancient system by which cells could sense a molecule that was a bellwether of viral infection (dsRNA) and respond by producing a signal that would tell the organism to dedicate its efforts and energies toward fighting viruses.

Early studies of systemic immunity were by no means limited to animal cells. Even as the first observations of an "interferon" response in animals were made in the 1930s, it had already been observed that plants could induce some remarkable immune responses. Applying a virus in one area of a plant could yield viral resistance (at least in some cases) that extended throughout the plant [e.g., 18,19]. Although these experiments indicated that plant had an immune system, it was known that they lacked the specific immune components (including antibodies and white blood cells) that had been studied for many years in animals.

This historical context of the gene silencing field thus includes the early recognition of an animal immune response (albeit a general one) dependent on double stranded RNA, and a plant immune response (albeit with trigger unknown) that could disseminate a specific signal over substantial biological distances.

GENE SILENCING ASSAYS IN A CONVENIENT NEMATODE

Now it's time to introduce another lead character into our story, one that is a close friend to Craig, myself, and to a few thousand other researchers worldwide. *Caenorhabditis elegans* is a nematode roundworm about 1 millimeter in length. In this lecture series, there were three talks on *C. elegans* in 2002 by Sydney Brenner, John Sulston and Bob Horvitz. Dr. Brenner credited "the worm" as deserving a significant portion of the scientific accolades (although he was reluctant to provide a monetary share to the worm) [20]. We should certainly credit this beast as well: *C. elegans* has turned out to be a very fortunate choice for studies of gene silencing. As you will see, the worm's vehement responses to foreign information have provided first great frustration and later some valuable insights.

One of the aspects of *C. elegans* that Craig and I have been very pleased with is the ability to microinject macromolecules (DNA, RNA, protein) into the animal [21–25]. Figure 2 is a picture that Craig took of this process, showing a fine glass needle injecting solution into an animal. After the needle pierces the cuticle, pressure is applied and some of the fluid comes into the cell that is being filled. The cell being injected in this photo is the germline or gonad of the worm, a large cell with hundreds of individual nuclei surrounding a common core of cytoplasm. Each gonad will generate hundreds of oocytes, making this is a remarkable technique for being able to influence a large population of animals with just a single microinjection. The microinjection needle can be filled with almost any liquid including the great variety DNAs, RNAs, and proteins that we can now design and synthesize in the lab. The simplicity of microinjection for *C. elegans* provided an enticing experimental tool to manipulate the genome of the organism and observe the consequences to developmental events and physiology. At the same time, this technology has allowed a number of us in the field to study the diverse responses this system has to foreign information.

Among the goals pursued in early applications of *C. elegans* microinjection was to turn down or turn up gene expression for specific genes. In the mid 1980s, as a Helen Hay Whitney Fellow working at the Medical Research Council Lab of Molecular Biology, in Cambridge UK, I had begun doing experiments toward this goal, using among other tools the *unc-22* gene that provided some of the first characterized DNA clones for the worm. It was already known through some very nice classical genetics that reducing expression of *unc-22* led to a movement defect, a twitching behavior that is very characteristic of alterations in the activity of this gene [26,27]. Don Moerman, Guy Benian, and Bob Waterston prepared fragments of *unc-22* [28] that I then injected with the hope that the injected fragments might recombine with the normal *unc-22* allele and produce a loss-of-function character that could then be studied. The results of these experiments were a puzzle: although twitching worms appeared in populations derived from the injected animals, there was no direct alteration in the original *unc-22* gene. Instead of the sought-after recombination event, it appeared that the

Figure 2. Micrograph of microinjection needle delivering a solution of DNA to the gonad of a *Caenorhabditis* adult hermaphrodite. Left, Microinjection needle poised at the side of the worm. The needle is filled with a solution for injection and is kept under a slight positive pressure until it is inserted into an animal (middle) whereupon an increase in pressure leads to microinjection of a volume of the material from the needle. After this, the needle is removed and the cuticle of the animal quickly recovers. Photographs courtesy of Dr. Craig C. Mello and reprinted from Mello and Fire, 1995 [25].

presence of extra DNA from the *unc-22* locus could induce the worm to turn down expression of the endogenous *unc-22* gene [29]. Several explanations for this unusual suppression effect seemed reasonable at the time: perhaps the endogenous *unc-22* locus DNA somehow paired with the foreign copies of this DNA; perhaps the foreign DNA was a template for synthesis of some amount of antisense RNA, which would then neutralize the activity of the normal transcript by base pairing, perhaps the fragments of *unc-22* were producing an aberrant protein or binding an essential regulatory factor, and perhaps there were some other mechanisms that were yet to be recognized.

Regardless of the actual mechanism of the interference in these initial experiments, the antisense strategy for "targeted" disruption of gene expression seemed particularly worthy of an explicit test. Such strategies were by no means novel at the time, having been pioneered some years earlier by Zamecnik and Stephenson [30], and by Izant and Weintraub [31]. In 1987, just after moving to Carnegie Institution in Baltimore, my co-worker Susan White-Harrison began to build DNA constructs to perform such an explicit test. Susan's constructions relied on our ongoing elucidation of muscle promoters (DNA sequences that instruct RNA polymerase to begin RNA synthesis in muscle cell nuclei). We expected a promoter hooked up to an *unc-22* fragment in the "antisense" orientation to give antisense RNA and thus perhaps gene silencing while the corresponding "sense" construct would give at most an excess of the sense strand and thus no expected silencing. We were hardly surprised when the antisense constructs produced a targeted interference effect (knockdown of the corresponding endogenous gene). This was consistent with a substantial number of reports of successful antisense intervention already in the literature. We were very surprised, however, when the control 'sense' constructs produced a similar interference effect [32,33]. The assumption for the "experimental" construct was that the antisense RNAs were finding their sense equivalents by standard Watson-Crick base pairing and taking the sense RNAs out of circulation. So what was going on

with the sense constructs (where if anything, we might expect the fragment inserted into the expression vector to be over-expressed)? Although this mystery was intriguing, it was hardly compelling at the time. The propensity for DNA transgenes to produce unwanted RNA transcription was certainly a good starting point for potential models, and a reasonable explanation (that somewhat dampened any immediate research on our part) would have been that the transgenes for some reason produced sufficient antisense RNA to yield an interference effect.

A significant milestone in the study of silencing in *C. elegans* was the demonstration that direct RNA injection could induce an interference effect [34]. This observation came from work of Su Guo, who at the time was a graduate student in Ken Kemphues' lab at Cornell. Sue's insight that injection of RNA might provoke silencing turned out to be correct. Moreover, she was able to demonstrate effects with either sense or antisense preparations of RNA. This set of experiments had two lessons. First, the experiments established a remarkably efficient means of disrupting gene activity (particularly in embryos), thus facilitating a wealth of experiments in what we now call functional genomics (efforts to assign function to genes that are discovered by large scale sequencing). Second, the mystery of the interfering sense preparations was accentuated since 'sense' RNA preparations could still trigger an interference response.

After Su's experiments established RNA-triggered silencing as both a mystery and a powerful technique for studying gene function in the embryo, several other groups started working with the technique and marveling at it's unusual character. Craig Mello, first as a postdoctoral fellow working with Jim Priess at the Fred Hutchinson Cancer Research Center, and then as a new faculty member at the University of Massachusetts, began in particular to apply the technique [35] and to study the phenomenon as a window on a fascinating fragment of the tapestry of biological regulation. As I will describe later, a significant advance in understanding the concerted nature of the response came when Sam Driver and Craig discovered that the silencing could be evoked by a diffusible and specific molecular signal. As the experience from Craig's group and others with this odd form of gene silencing accumulated, much of the information was shared with the *C. elegans* community. Although the name "antisense" had initially been used to describe this process, it was clear (from the 'sense' results) that the phenomenon was not a simple one of antisense occlusion. There was thus a need for a new designation for the process, and after putting a few potential names to a vote, Craig chose the term "RNAi" ('RNA interference') to refer to the observed silencing process(es) [35].

TOWARD A STRUCTURAL UNDERSTANDING OF THE RNAi TRIGGER

For my perspective at the time (at that point as an observer of work in other labs on the worm's response to injected RNA), much of the accumulating

data came together at an informal discussion on RNA-triggered silencing organized by Craig at the 1997 *C. elegans* meeting in Madison, Wisconsin. The workshop was held in the theatre of the Student Union, with the normal capacity of the room overwhelmed (I was sitting on the floor). At the time, there were several very clear but also very unexplained features of the response. In addition to the diffusible signaling data (reported by Driver at the previous year's *C. elegans* meeting), and the ability of both sense and antisense strands to produce the interference effect, there was a remarkable persistence to the effect. From work of Craig, Rueyling Lin, Morgan Park and Mike Krause, and from Patty Kuwabara [36], it was clear that injected RNAs could have effects for several days after the injection occurred (and in some cases generations after the initial injections). This contrasted with observations that Geraldine Seydoux had made several years earlier [37], showing that many native RNAs were comparatively unstable during the same time period in the same cells. The confluence of these two results suggested perhaps that the active interfering material had some kind of a privilege in its stability. Perhaps the injected material contained a fraction of particularly stable molecules that were responsible for the persistent interference.

Double stranded RNA was known to be relatively stable both chemically and enzymatically [e.g., 38]. In addition, dsRNA was a known low level contaminant in synthetic RNA preparations [e.g., 39]. From my graduate work with RNA polymerases, I was certainly also very familiar with the sometimes annoying ability of RNA polymerases to start in vitro at ends and other fortuitous sites. Thus the concept that double stranded RNA might be a component of the injected material was hardly a leap of logic. Arguing strongly against dsRNA as a potential effector was the fact that native dsRNA would have no free base pairs to interact with matching molecules in the cell. Thus a rational first guess would have been that injected dsRNA would have been unable to interact specifically with cognate sequences and thus rather useless for triggering genetic interference. A critical review of my research plan coming out of the 1997 worm meeting would certainly have brought this up as a major concern. One could imagine (in retrospect as well as currently) many different models and explanations for the phenomena. Some scenarios would have spawned interesting experimental investigations while others would have been of only limited interest; I was certainly fortunate that our research grant was not up for renewal for at least a few months.

The strength of the experimental system with *C. elegans* was that virtually any biochemical sludge could be concocted and injected into a worm, with a very rapid (and in most cases quite specific) assay at the end for targeted genetic modulation. This made it possible to test somewhat far-fetched hypotheses (like the involvement of dsRNA) without spending years or "breaking the bank". A second ingredient in testing the double stranded RNA was someone to make the experiments happen. SiQun Xu, with extensive experience with both nucleic acid synthesis and isolation and with *C. elegans* microinjection, was certainly the ideal person for this for many reasons. The setup was particularly comfortable for me since SiQun could thus do the syn-

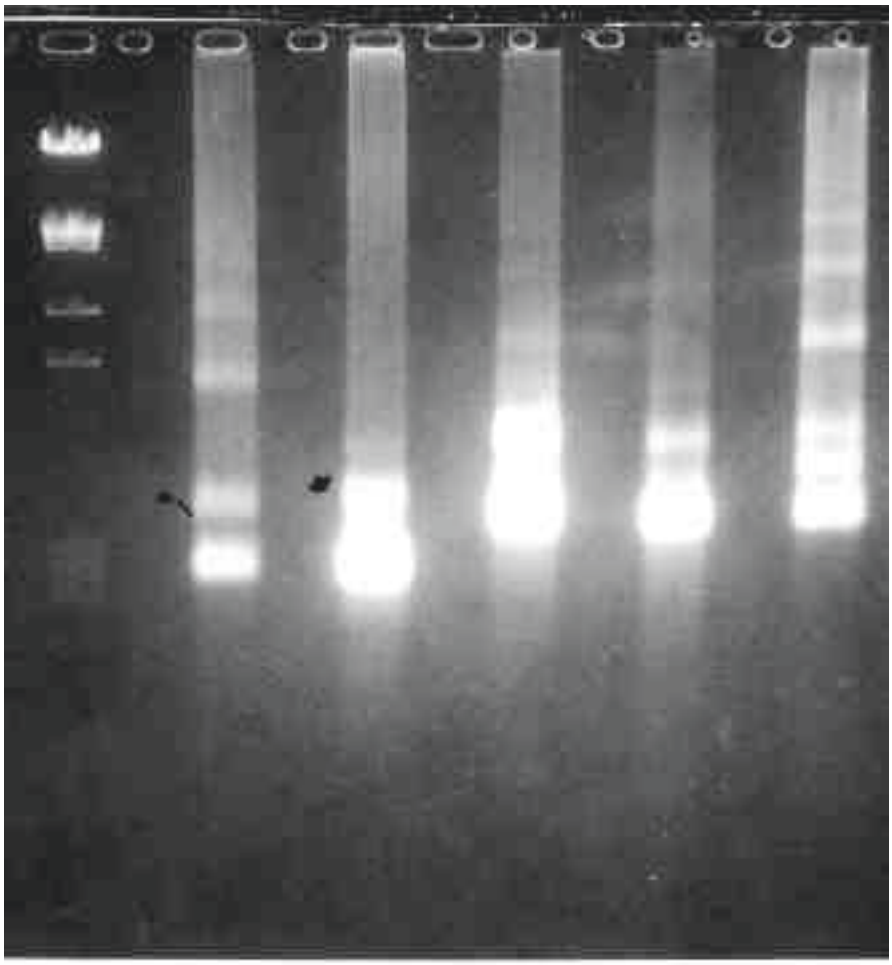

Figure 3. Electrophoretic separation of RNA prepared by *in vitro* synthesis. Left lane, marker DNAs. Remaining lanes show RNA populations with a strong band (bright signal) in the expected position for single stranded sense or antisense RNA (depending on the intended synthesis) and a number of unexpected bands (and a smear) in each lane that is visible due to overexposure of the photograph. RNA is resolved on agarose gels and visualized by fluorescence upon interaction with the included dye Ethidium Bromide. Source: Original Gel Photograph, SiQun Xu and Andrew Fire, 1997.

theses and injections and I just needed to visit my microscope in the lab for an hour or two every day to look at the injected animals and their progeny.

SiQun first repeated the kinds of RNA synthesis reactions and injections that others had done, using in this case our favorite gene, the *C. elegans unc-22* gene. This of course worked, generating a bunch or twitching worms as evidence for effective silencing of endogenous *unc-22* activity and setting the stage to use this assay in characterizing the relationship between structure and interference of the injected RNA. The picture shown in Figure 3 shows a series of the initial RNA preparations resolved using an electrophoretic

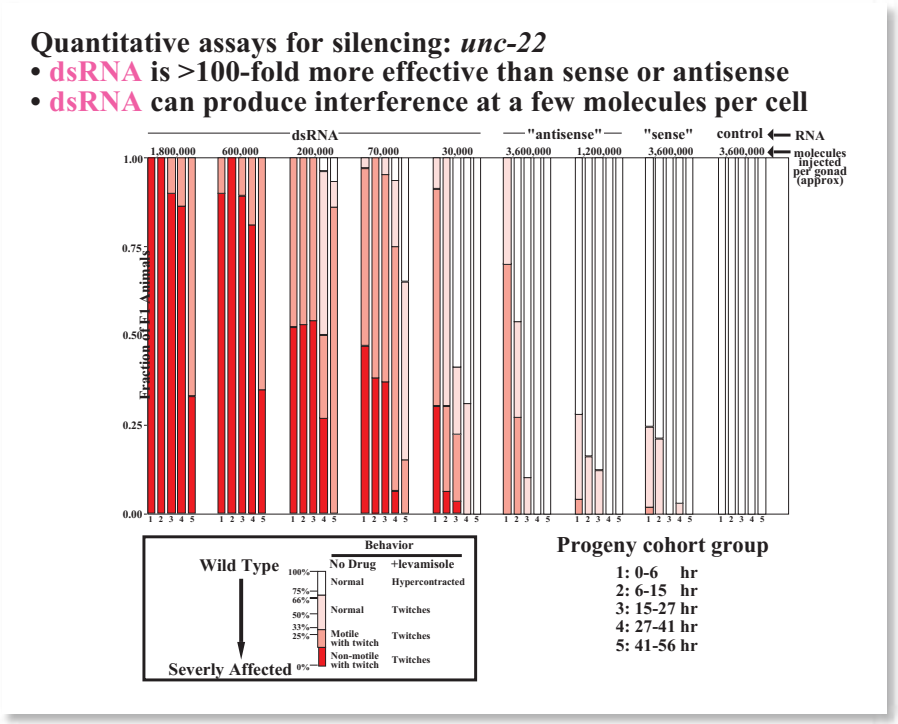

Figure 4. Quantitative assays for silencing of *unc-22*. Preparations of RNA similar to those in Figure 3 were enriched in the expected (sense or antisense) species by excising the major bands from agarose gels and extraction of RNA. Some unwanted dsRNA may persist in these samples but in general at a greatly reduced level when compared to samples not subject to purification. Individual sections of the graph show biological responses following injection of differing concentrations of single stranded and double stranded RNAs as diagrammed below (more highly affected animals are shown with a more intense red color). [Source: Reference 40 Supplement; see reference also for additional details].

field and an agarose gel. What you can see is a very prominent band, a bright spot, where the RNA that we expected was. This photo was deliberately overexposed to reveal any other components that might be present, and one can certainly see additional (minor) bands and a general ”smear” in addition to the major (expected) bands. After a few preliminary explorations of the dsRNA hypothesis using this assay with these impure RNA preparations, I was somewhat encouraged but still be no means convinced. It was clear that a cleaner preparation of starting material was needed. To achieve this, SiQun cut out the major bands from this gel, extracted the RNA and injected the purified sense or antisense RNAs into worms. This produced a result, albeit negative: almost all of the activity was lost by purification of single strands, suggesting that the sense and antisense weren’t the material that was causing the interference.

SiQun’s purified strands also provided a better starting point for testing the dsRNA hypothesis, since the two nearly-inactive strands could be mixed

in a test tube to produce a well defined double stranded product. SiQun's injection of double stranded *unc-22* RNA formed in this way produced a remarkable result, with all of the resulting animals twitching strongly. To see how potent the effect was, SiQun injected smaller and smaller amounts of the double stranded material (Figure 4). The resulting animals showed an interference effect even after substantial dilution. When we finally did the calculation of how much material was being injected, we realized that we were seeing effects down to a few molecules of the double stranded RNA per cell. This was remarkable in that we knew from some previous work that we and Don Moerman and others had done that the target *unc-22* mRNA was much more abundant.

As with any uncharted phenomenon, the first job of the scientist is to look for explanations based on known processes. The summer of 1997 was a busy one for phone lines, email connections, and delivery services between Baltimore and Worcester, with numerous collaborative experiments with Craig and SiQun now joined by Steve Kostas and Mary Montgomery. In addition to the characterization of the specificity/generality/character of the effect on target genes, a major goal was to definitively ask whether double stranded RNA in the interfering sequence was directly responsible for the observed effects. An alternative explanation was still quite tenable: that double stranded RNA produced a non-specific response (either local or global) that potentiated the activity of small amounts of antisense. Settling this issue took a bit of molecular artistry to pursue. The most satisfying were a set of assays where we could look at gene-specific interference by complex RNA molecules that contained single stranded RNA matching one gene and double stranded RNA matching a second gene. All of these experiments pointed clearly to induction of specific interference by regions of double stranded RNA, and by the end of the summer we were all felt that a paper could be submitted definitively describing the ability of dsRNA to trigger a gene-specific and systemic silencing process [40].

dsRNA-TRIGGERED SILENCING PROCESSES AND THEIR ROLES: LESSONS FROM WORMS, PLANTS, FLIES, FUNGI, AND OTHER SUNDRY BEASTS

But of course we still did not know what was actually going on, in particular what was actually happening to the expression of the target gene. Mary Montgomery was certainly in an excellent position to pursue this question, having spent several years working around the apparent reluctance of *C. elegans* to translate injected RNA. The idea of an RNA injection experiment with a dramatic consequence (albeit strange and unexpected) was certainly enticing, so she took up the question of what happens to gene expression in the presence of injected double stranded RNA. At the time, one could imagine the interference affecting any step of gene expression or cellular homeostasis. Mary had observed that target genes lost their ability to accumulate mRNA in the cytoplasm [40]. Extending this analysis, she was able

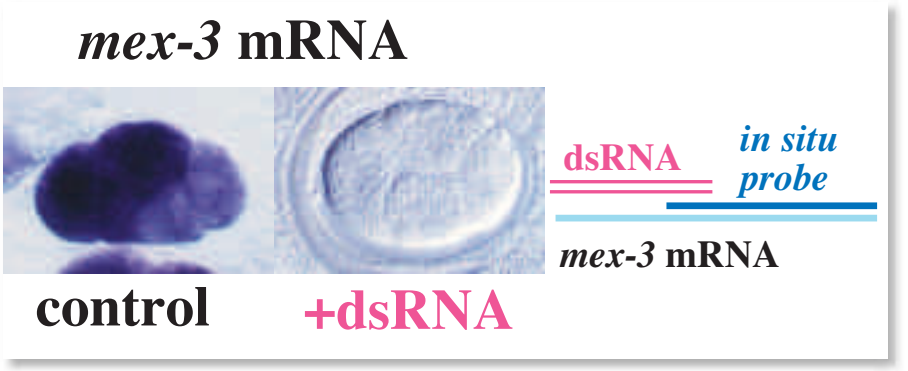

Figure 5. Injection of dsRNA results in disappearance of the targeted message. This experiment (from Mary Montgomery [40]) shows embryos of *C. elegans* with and without dsRNA injected corresponding to the mex-3 gene [142]. In control samples a strong signal is observed on *in situ* hybridization [AA] (intense blue stain, left panel), indicating a high level of mex-3 transcript throughout the four-cell embryo. Following dsRNA injection, the mex-3 transcripts are not detected (Center Panel).

to demonstrate that RNAi was accompanied by destabilization of the target mRNA in the nuclei and cytoplasms of infected cells [41]. In some ways we were lucky be working on one of the simpler dsRNA response systems; current knowledge of RNA-modulated gene expression has led to the realization that virtually every activity of genes can be affected by modulatory RNAs (replication, DNA structure and sequence, chromatin structure, transcription, processing, localization, ability to engage the translation machinery, and translational progression [e.g., 42–48]). Mary's experiments also provided a remarkably graphic description of the effectiveness of RNA interference in *C. elegans*. Figure 5 shows an example of this, with a test gene examined with and without interference at the level of messenger RNA abundance. In the case of a control sample, the messenger RNA for this gene is highly abundant and readily detected by the color reaction derived from a procedure called *in situ* hybridization [49]. After interfering with the test gene by injecting the corresponding dsRNA the messenger RNA was essentially undetectable.

The hypothesis that came from Mary's experiments was that the double stranded RNA produced a condition where the target transcript was produced but was very unstable. Restated, this postulates a sequence-specific RNA degradation system that could be triggered by dsRNA. An old TV show called "the twilight zone" was based on the idea that the universe contains many phenomena that go beyond our capacity to understand. As of early 1998, the data we'd accumulated was certainly consistent with the hypothesis that we were at least temporarily in the "twilight zone".

Accentuating this sense of unexplainable phenomena was a series of tests on the spatial requirements for dsRNA administration. These observations had a very rational starting point. When Su Guo did her original RNA injections at Cornell, she had intended to test for a biological effect of the injected material in the gonad. So she injected the gonad and indeed an effect

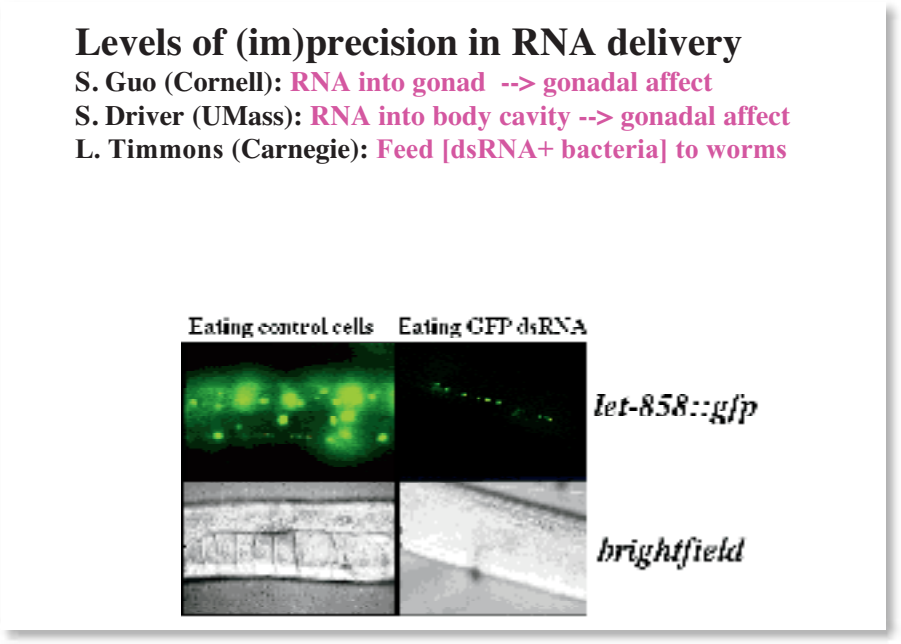

Figure 6. RNA delivered outside of a cell can produce a potent interference effect. Above, schematic diagrams of RNA delivery experiments from Su Guo and Ken Kemphues [34], Sam Driver and Craig Mello [40], and Lisa Timmons [53]. Below, examples of feeding-based RNAi. Both animals are from a *C. elegans* strain where generalized somatic expression of a green fluorescent reporter is readily observed. The animal at the right is fed on bacteria expressing dsRNA corresponding to the gfp coding region. The animal on the left is fed on bacteria not expressing this construct. Note the dsRNA-dependent loss of gfp activity in this example in all visible cells except those of the nervous system.

was seen there [34]. The science/life-lesson that one can draw from this is "if you can do the experiment the way that seems most likely to be effective, do it just that way".

A subsequent observation from Sam Driver and Craig Mello, yields the lesson "if you can't do the experiment the way that seems most likely to be effective, still do it". In 1996 Sam was a beginning graduate student in Craig's lab at the University of Massachusetts. He was just starting out with injection and so putting the needle into the correct tissue was problematic. Sam and Craig realized that despite the improperly placed needles, the injections were still producing extremely efficient interference. When they then deliberately injected into the "wrong" place (the body cavity), they still observed a strong biological effect. Later, Craig and SiQun Xu each extended this set of observations to an extensive list of tissues where dsRNA injection produced a systemic effect.

Finally, we have a third lesson, this time derived from experiments initiated by Lisa Timmons, then a postdoc in my lab at Carnegie and now a faculty member at Kansas University. The lesson here, if you're a postdoc or perhaps a graduate student, is to do experiments that your advisor would never con-

done or suggest. Lisa engineered *E. coli*, which is a bacterium that is the food source for *C. elegans*, to produce double stranded RNA. When she fed this genetically modified food to the worm, she saw a gene-specific interference effect. Figure 6 shows a case where she had engineered the bacteria to make double stranded RNA corresponding to the fluorescent reporter GFP (a wonderful tool for following gene expression and cell patterns during development) [50–52]. Starting with a worm strain that produces GFP in essentially all somatic cells, Lisa found that the ingested RNA could silence gene expression throughout the animal [53]. (The picture tells another interesting story, which is that there is considerable resistance to RNA interference in nerves of the animal. Although we have yet to understand the basis or reason for this, the wholesale alteration in the efficacy of the pathway in different tissues provides additional evidence for a very deliberate biological process.) Hiroaki Tabara, a postdoctoral fellow working with Craig at the time, went even beyond the "feeding" experiment, showing that simply soaking worms in double stranded RNA could produce an interference effect [54]. These experiments were particularly surprising given our expectations that cell membranes would block all but the smallest diffusible molecules from moving between cells. We knew that there was little or no diffusion of DNA. A theme in macromolecular transport of large charged molecules has been that the cell transports only things that might be useful, with those transport mechanisms very specific and well controlled. I certainly had no idea of why the worm would be transporting dsRNA-derived signals in a facilitated manner.

So now we had every reason to think we were in "the twilight zone". Despite this, we were certainly pleased at our accomplishments in contributing to the development tools for manipulating gene expression in *C. elegans*.

We now step through a doorway from the limited world of our favorite model organism to the much richer real world inhabited by species too numerous to count. This transition is accompanied by the recognition that discoveries that we may initially view as our technical "accomplishments" are invariably a reflection of underlying processes that are a natural part of sustaining life.

Soon after the initial description of dsRNA-triggered silencing in *C. elegans*, several descriptions of similar processes appeared for other groups of organisms. These initially included observations from *Drosophila* (a fruit fly), Trypanosomes (single cell parasites), and plant systems [55–58], with many other organisms rapidly joining the list. Mammals were conspicuously absent from the initial list of organisms generally amenable to this type of manipulation. The exclusion of mammals from the list of easily manipulated species was not a surprise: the non-specific responses to dsRNA that were originally discovered by Hilleman and colleagues [14] were certainly sufficient to confound any analysis of specific genetic interference. Nonetheless, early efforts in this area provided both an indication of the potential existence of specific dsRNA responses in certain specialized mammalian cell systems (e.g. oocyte and ovary cells [59–61]) and of the predominance of the non-specific response in most others [e.g. 62].

In addition to establishing a broader biological occurrence of dsRNA-triggered genetic interference, the demonstration of dsRNA-triggered silencing in plants and fungi illuminated the process by connecting our rather fragmentary observations from *C. elegans* with a broad gene silencing literature. Indeed, papers starting a decade earlier from fungal and plant systems had been the first to describe sequence-specific effects of foreign DNA transgenes on the corresponding endogenous genes [63–67; also see ref. 68]. Intensively creative work had allowed workers in both plant and fungal fields to track down the sequence-specific foreign DNA reactions as a complex set of responses that could independently attack the target gene's chromatin or RNA [e.g., 46,69–71]. The distinctive spatial patterns of silencing for endogenous genes in plants [65,66] had been one of many features that had drawn a small cadre of highly innovative investigators to study this question for its own sake. Demonstrations of a systemic signal in the plant silencing [71,72] were particularly striking and certainly led to a clear recognition of potential similarities between the phenomena that had been observed in *C. elegans* and gene silencing in plants.

At this point, it is worth pointing out the substantial advantages of studying gene silencing (or any other important phenomenon) in more than one model system. The advantages of studying silencing in *C. elegans* turned out to be the flexibility of designing and making arbitrary RNA structures in a test tube and delivering them easily (by microinjection) into a rapid assay system (the nematode). This had circumvented many of the challenges faced by researchers working in plant systems, where such capabilities were not straightforward and complex issues of transgene structure and transcription confounded initial attempts to definitively assign a specific RNA structure as the trigger for the response. On the other side of the balance, plant systems offered a remarkable means to investigate the biological role of the interference response. Starting with the earliest recognitions of transgene-derived viral resistance [73] and observations that viral RNAs could be both triggers and targets for the silencing [46,74,75], it was rapidly clear that the silencing system might serve in the natural protection of plants from "unwanted information" in the form of viral pathogens. Definitive demonstration of this point came from a number of analyses of virus/host interaction.

To be a successful, one would expect a proposed antiviral system to effectively block pathogenesis of at least a subset of viruses that might otherwise menace the organism. Since it is well known that viruses still succeed in the world (much to our dismay), there must also be ways in which the virus can counteract any cellular defense mechanisms. A critical point in defining the role of RNA-triggered silencing process was the recognition that many successful plant RNA viruses produce protein components dedicated to the inactivation of the silencing mechanism [e.g., 76–79]. Deliberate suppression of host RNA-triggered silencing responses allows viral infectivity in at least a subset of plants for any given virus. The balance between the silencing mechanism and viral attempts to subvert it forms the basis for an ancient "arms race" between virus and plant. The character of this arms race

was further evidenced in these studies by the ability to generate attenuated virus (by removing the anti-silencing function) and hyper-susceptible plants (by expressing a relevant viral anti-silencing protein or interfering with the endogenous RNAi machinery).

The emerging recognition that the transgene response mechanisms in plants were at least in part an antiviral response had raised the compelling question of how viral activity could be specifically recognized by a silencing apparatus. A rather remarkable proposal to explain this was put forth by Ratcliff, Harrison, and Baulcombe in mid 1997 [80], in a paper that arrived at Carnegie just as we had scored our first assays to test for the ability of dsRNA to trigger gene silencing in *C. elegans*. Baulcombe and colleagues had reasoned that unique features of viral replication intermediates might lead to improved transgene-based triggers for gene silencing, stating "It may be possible to increase the incidence of gene silencing by ensuring that transgene transcripts have features, such as double-strandedness, that resemble replicative forms of viral RNA" [80]. Combined with experiments suggesting an association between silencing effectiveness and certain secondary structures in the transgene and transcript [68,82], these proposals would almost certainly have inspired similar experiments to ours. The confluence of the two approaches, as always in science, proved to be the most powerful driver of further work, as the combination of chemical definition of the trigger in *C. elegans* and a biological explanation of its efficacy in plants led to a rapid explosion of scientific effort in the area.

TOWARDS A REACTION MECHANISM: EFFORTS TO PEER INSIDE THE BLACK BOX

Despite the great enthusiasm from those of working with plant, worm, and insect model systems, the mechanism by which dsRNA could silence gene expression was still an unknown. Seminar slides made at the time would show dsRNA and the mRNA target somehow entering a large and mysterious "black box", followed by degradation of the target RNA and some unknown fate for the effector dsRNA. This "black box" explanation limited our grasp of the RNAi system, both for understanding the underlying biology and for applying RNAi to organisms (like humans) where the response to dsRNA was more intricate than for "simple" invertebrates. The key questions (both in terms of molecular mechanism and in terms of potential roles of RNA-triggered gene silencing as an immune process) revolved around a need to understand the structure of the molecular assembly responsible for recognition of the target message by the effector RNA. Like antibody-antigen complexes in classic immunity, the identification of a "fundamental unit of recognition" seemed a key step in elucidating RNAi-based immunity in cells.

Some of this work could be done using *C. elegans*, and I will describe this in a bit of additional detail. Keep in mind (and I will describe at the end of this section) that much of the ongoing work was at this point being pursued in parallel in different systems by a plethora of research groups each with

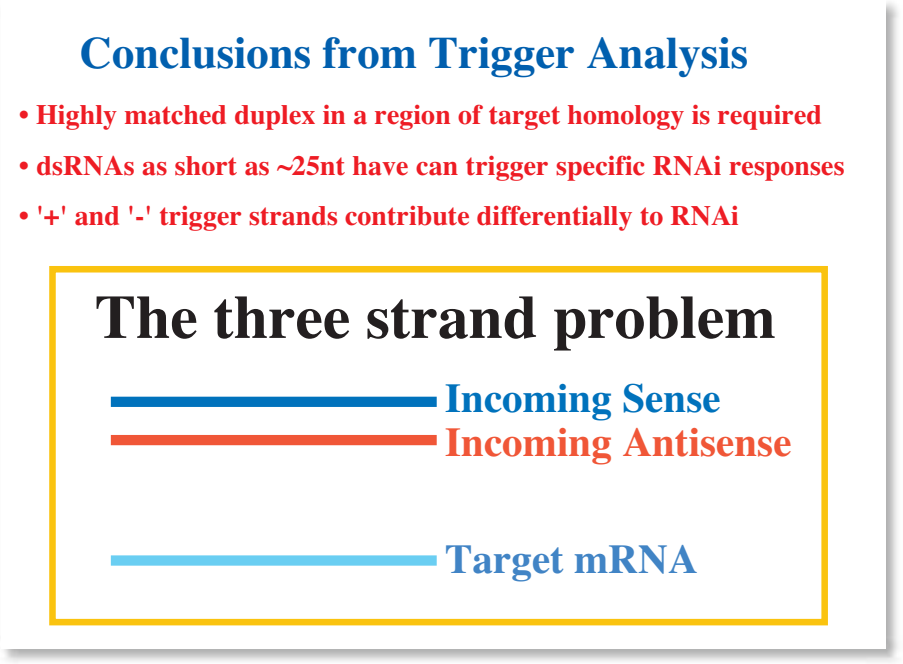

Figure 7. Conclusions from experiments where RNA interference was assays after structural and chemical modifications had been made in injected RNAs. For details see text and Parrish *et al.*, 2000 [83].

their own angle on a specific model organism and interference assay. RNAi is a three strand process [Figure 7] involving a sense strand and an antisense strand in the trigger and a target transcript in the cell. We could manipulate the trigger strands extensively in an attempt to determine exactly what was required for the induction of specific interference. This analysis gave several specific results [83]. First, we found a different set of chemical requirements for the sense and the antisense strands in inducing interference. Second, there was a rather stringent requirement for sequence matching between the two trigger strands and with the target strand. Third, although there was a decrease in effectiveness as we used shorter and shorter triggers, we could obtain a response in *C. elegans* with triggers whose length was in the 20s of nucleotides. Combined with complementary structure-function experiments carried out at a similar time in other systems [e.g., 84] these data evidenced a very concerted chemical precision of effector RNA recognition and action in the (at that point still very unknown) black box.

A second area in which *C. elegans* could readily contribute to understanding of RNA-triggered silencing revolves around a genetic screen. The screen, originally executed by Hiroaki Tabara and Craig Mello [85], involved an important modification of Lisa Timmons' feeding experiment. Hiroaki engineered *E. coli* to produce a specific dsRNA, but in this case the dsRNA was targeted toward an essential gene in *C. elegans* (a gene called *pos-1* that

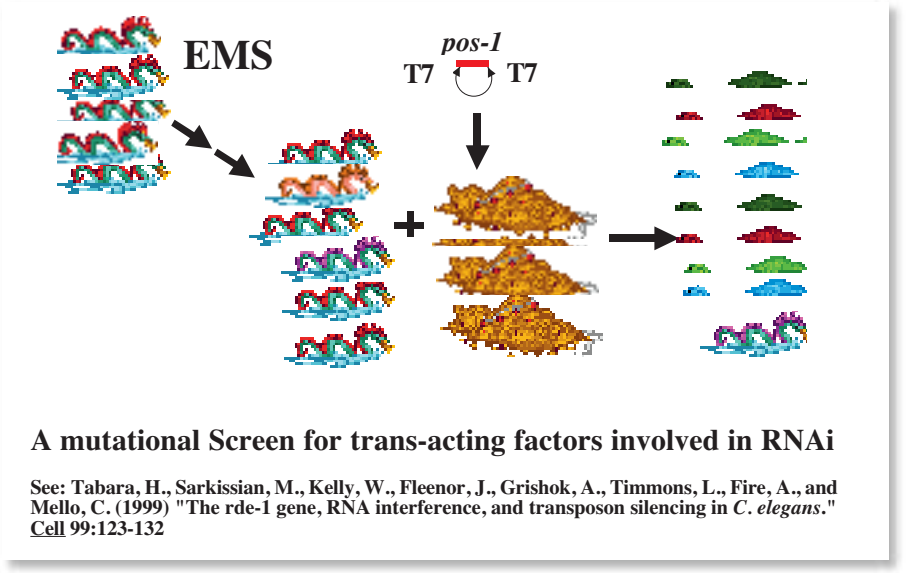

Figure 8. Identification of mutations that eliminate responses to foreign RNA but are compatible with life for the worm. After mutagenesis with the chemical mutagen Ethyl Methane Sulfonate [26], animals were grown for several generations and then transferred to an *E. coli* food source expressing dsRNA corresponding to the *C. elegans pos-1* gene. Embryogenesis is arrested in the vast majority of the resulting population and only mutants such as those eliminating RNAi can continue growing as a population. For details see text and Tabara *et al.*, 1999 [85].

Hiroaki had characterized during his graduate work with Yuji Kohara [86]). Without the activity of this gene, worm populations could not survive, so that the engineered bacteria are an exceedingly poor food source for *C. elegans.* Selecting the extremely rare animals that can grow on that food source was then possible [Figure 8] and was facilitated by working with populations that had been chemically treated several generations earlier to produce mutations. Among the animals that grew on this food source were a subset that lacked the responses to all the kinds of foreign dsRNA that we had used for interference. For at least two genes, Hiroaki found that a complete loss of function resulted in a worm that looked normal (or nearly normal) in the laboratory, but which was unable to respond to our dsRNA challenges. The existence of these mutations provided further (and very compelling) evidence that RNAi was a concerted process. If the ability of dsRNA to silence genes had been a simple reflection, for instance, of the physical chemistry of dsRNA, then we would have been unlikely to find mutations that abrogated this activity. That *C. elegans* could survive without the process and grow normally (at least in the artificially pristine conditions of an isolated Petri plate) was a demonstration that the organism relied on a dedicated mechanism to facilitate dsRNA-triggered silencing. Through considerable effort, mostly from Hiroaki and Craig, it was possible in a relatively short time to identify

the genes which had been mutated in the resistant strains. The identities of the corresponding gene were both illuminating and frustrating.

rde-4 encoded a protein with a structure clearly suggestive of an ability to bind to dsRNA [87]; although certainly reassuring, this identity by itself (and the expected ability of the protein to bind dsRNA non-specifically [88]) was not sufficient to illuminate the underlying mechanism.

rde-1 encoded a protein from a large family (now called the "Argonaute" family) for which there was at the time only a trace of biochemical data. Proteins from related families had been shown to play key developmental roles [89–91]. There was some indication of an RNA interaction [92], but there was little biochemical information beyond this. As it became clear that other genetic model organisms shared a dsRNA response mechanism, it likewise became clear (from genetics in plants, fungi, and flies [e.g., 93–95]) that at least a subset, like *C. elegans*, could survive without this mechanism. The ability of diverse organisms to encode proteins of similar character to those involved in *C. elegans* gene silencing, and the eventual identification of homologous genes as functionally required for RNAi in distinct model systems [e.g., 95–97] supported the argument that we were all looking at a similar and conserved biological process. Beyond the standard "model" organisms, the existence of homologous coding regions in mammals supported the argument that mammals might indeed also have similar responses if it were possible at some point to tease away the non-specific response.

Despite these hopeful suggestions, the RNA structure-function and genetic analysis had not put us in a position either to propose a unifying mechanism for RNAi or to design experiments to test for the efficacy of the system in mammals. Even in hindsight, going forward in either direction would have been complicated; in particular, the shortest RNAs that we had initially tested for interference in *C. elegans* [83] were too long to have fit into the what we now know as the RISC complex [see below], and were not of the proper structure to provide side-effect-free gene silencing response in mammalian cells.

Getting into the black box required a series of keen biochemical observations. I won't go into these observations in too much detail here, as the small RNAs that mediate exogenous and endogenous genetic control in diverse biological systems are certainly worthy of their own narrative. Still a summary of the small interfering RNA story serves to provide some context for how we now think about RNAi.

The first indication that a small RNA population might be key to the RNAi process came from experiments in plant systems that were carried out by Andrew Hamilton and David Baulcombe [98]. Studying plants undergoing experimental gene silencing, they found a population with a narrow size range of 21–25 whose presence was closely associated with the silencing. Critical to this analysis was the decision to look for RNAs in a small size range and the rather impressive chemical trick of actually detecting these RNAs.

With small RNAs identified as potential additional characters in the story, biochemical research gained considerable momentum. To know anything about what was happening in the black box required an ability to study the

reaction not within the complex environment of living cells, but in some type of isolated system. Two groups initially took up this challenge: one at MIT (Phil Zamore, Tom Tuschl, Ruth Lehman, David Bartel, and Phil Sharp) and one at Cold Spring Harbor (Scott Hammond, Emily Bernstein, David Beach, and Greg Hannon). Each succeeded independently (using very different approaches) in recapitulating the RNAi reaction in soluble extracts of *Drosophila* cells [99,100]. As the analysis of the biochemical reaction proceeded from these groups and others, it became clear that the small RNAs that Hamilton and Baulcombe had observed in plants were indeed central to the interference reaction. The reaction was, at least conceptually, divided into three phases, the cleavage of a long dsRNA trigger into shorter dsRNA segments, the loading of chosen single stranded products of this cleavage into a tight ribonucleoprotein complex, and the scanning of potential target RNAs in the cell by this complex [99–102]. The Hannon lab, perhaps while watching late-night television, coined catchy (and now standard) names for the two enzyme complexes central to the reaction: *Dicer* (which cleaves the dsRNA into short segments) and *Slicer* (which assembles around a single strand of processed effector RNA and goes on to cleave target messages [somewhat equivalent to the term RISC]).

The pathway that resulted from the confluence of biochemical and genetic analysis is shown graphically in Figure 9. The reaction initiates with cleavage of the large dsRNA fragment into small double stranded fragments. Selected strands of single stranded RNA then get incorporated into the "slicer" complex, which then searches around the cell looking for target RNAs in a manner that is not yet understood. When those target RNAs are found, they are cleaved by an enzyme activity which is intrinsic in the RISC, leading eventually to target degradation. Although this mechanism certainly didn't explain all of the phenomenology, it has proven remarkably general as a working model on which to base further study of RNAi.

Among the consequences of this model were some predictions of how to achieve specific RNAi in human cells. A key step in this was the detailed chemical description by Elbashir, Lendeckel, and Tuschl of the first small RNA intermediate in the silencing process [103]. This mostly-double-stranded small RNA population formed by Dicer had a characteristic set of termini with two slightly overhanging bases on each strand and the negatively charged phosphate group on the non-overhanging end. Recall that long double stranded RNA induced a nonspecific effect which prevented us from looking for any specific effects. Determination of the intermediate structure sparked an informed guess that RNAs of this structure, known from earlier studies to be too short to induce a strong non-specific response [e.g., 104], might produce a much more specific response. This was indeed the case, with reports appearing first from Elbashir, Tuschl and colleagues [105], and then in rapid succession from other groups including our colleagues Natasha Caplen and Richard Morgan at NIH [106]. The relatively straightforward nature of these assays led quickly to adoption of siRNA-mediated interference as a preferred method for certain analyses of gene function in mammals.

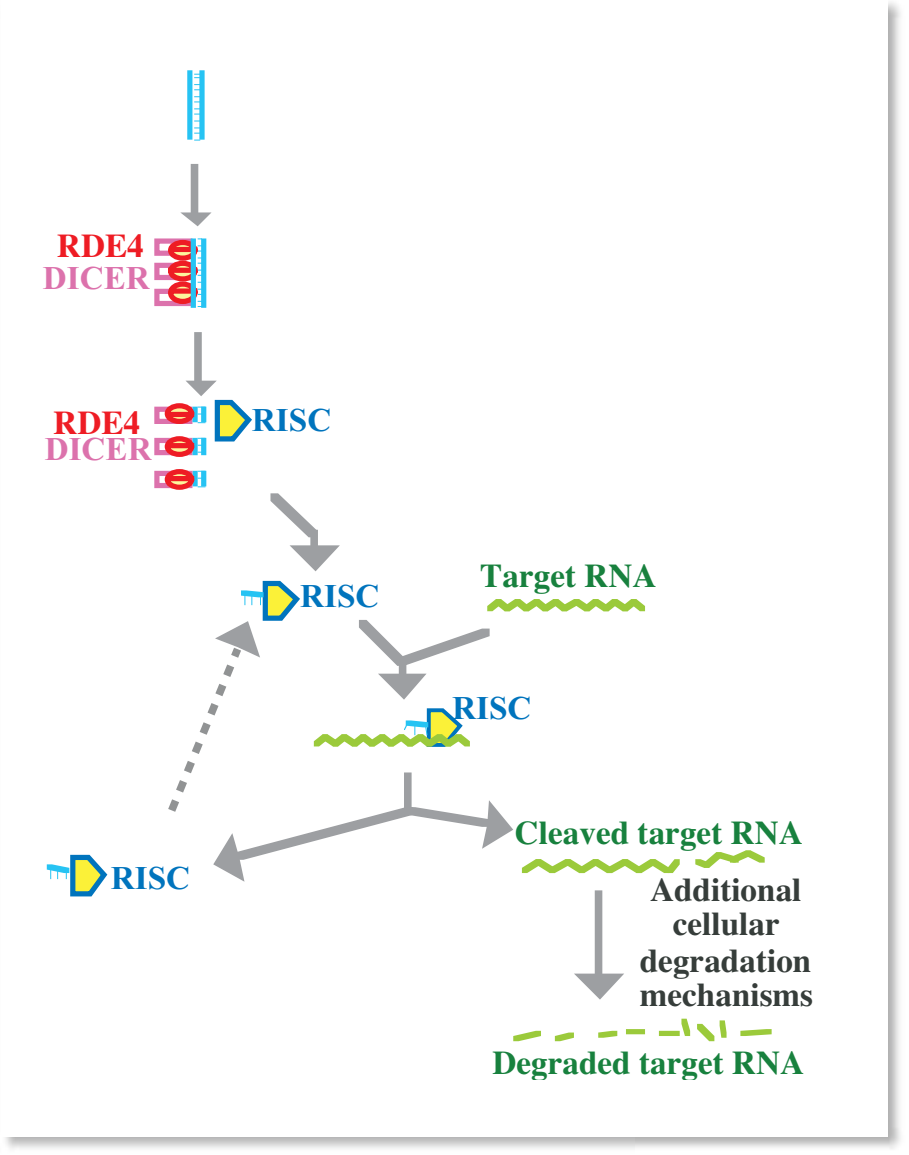

Figure 9. A basic model for the conserved central core mechanism in RNAi. Based on biochemical and genetic experiments as described in the text, double stranded RNA enters the cell, is set upon by a complex of a dsRNA-binding protein (RDE4 for *C. elegans*) and a dsRNA-specific nuclease (Dicer). Following dicing of the dsRNA into short double stranded segments, individual small RNAs are loaded into a second protein complex including a protein member of the *Argonaute* family (to assemble an RNA-Induced-Silencing Complex, also called a 'RISC' complex). These can then survey the existing RNA population in the cell for matched targets, which are then subject to degradation.

RNA INTERFERENCE AS IMMUNITY: SOME ANALOGIES AND QUESTIONS

The genetic and biochemical elucidation of RNAi also raised some interesting questions of analogy between the classic immune response (involving antibodies and lymphocytes) and RNAi (Figure 10). First will come the question of specificity. For the classic immune system, specificity is enforced by a series of interactions between recognition proteins (Antibodies and T-cell receptors) and their potential partners (including foreign proteins and other molecules). The flexibility of specific protein recognition repertoire thus serves as major basis for the classic immune response. For intracellular responses to foreign RNA, it appears that nucleic acid complementarity plays a similar role. Hybridization of short effector RNAs to a target message provides both rapid and specific recognition on which to base an immune response. The critical length of the duplex, in the 15–25 nt range, turns out from first principles to be optimal for achieving specific recognition without burdening the system by non-specific hybridization that would be more common with longer effector molecules (a point made many years ago by Tom Cech, in giving an introductory lecture in ~1991 to a group of scientists who hoped to use antisense technology for therapeutic goals).

A second challenge for the RNA interference pathway is how to ensure that no self-attack occurs that might harm the host cell; essentially there is a need to be sure that none of the cell's own essential genes are targeted by the RNAi mechanism. A part of this assurance relies on the use of dsRNA

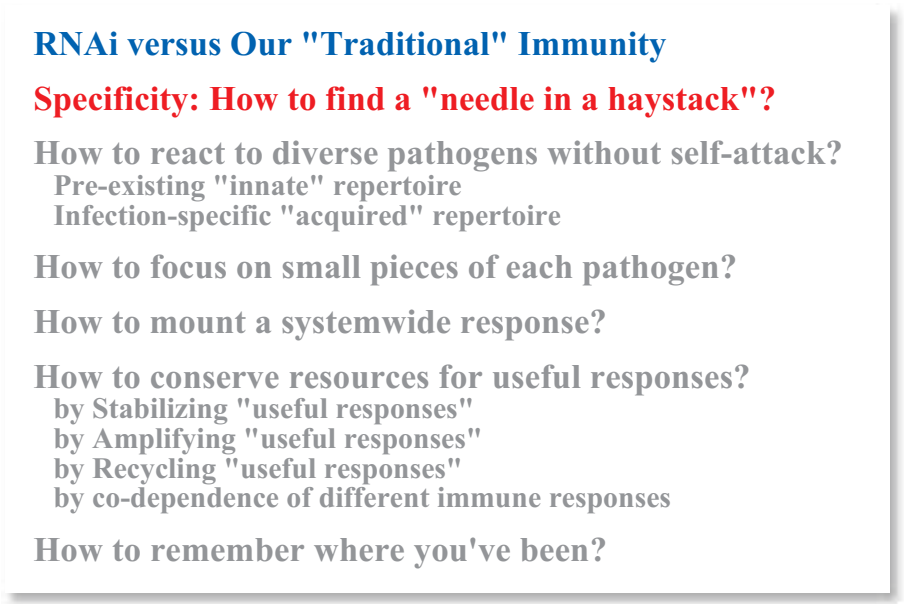

Figure 10. Points of comparison, analogy, and contrast between traditional immunity (T-cell/Antibody mediated responses) and proposed RNAi-based defense mechanisms.

as a trigger. Our cells don't normally use double stranded RNA to express our genes, they use single stranded RNA. Of course there may be cases where double stranded RNA is part of modulating gene expression, but for the most part, cells can avoid it if they need to. The interesting part of this avoidance is that it is evolutionary in nature. We presume that once the RNAi mechanism is in place, cells would evolve very diligently to avoid producing dsRNA in amounts that would shut off important endogenous genes. Any deviation from this could decrease the fitness of the organism, so over evolutionary time we expect a very effective avoidance of self-detrimental RNAi. This long-term mechanism differs from classic immunity in that the classic immune response avoids self-inflicted damage by a surveillance mechanism that (when everything is working properly) removes self-directed recognition elements continuously during the life of an organism. The consequence of this difference is that for RNA-based immunity it may be easier in real time to "trick" the system into targeting an endogenous component, something that could be an encouragement to the development of therapeutic strategies involving RNAi.

Breaking of the initial dsRNA trigger into small fragments reveals a third immune-related logic to the process. Certainly the dicing of the trigger serves to increase the number of independent molecules (and specificities) in the response, potentially providing a more effective trigger::target ratio for surveillance. In addition to this, the focus on short segments allows the system to respond to viruses that have mutated elsewhere in the genome but kept one or more essential sequences of greater than 20 bases. Finally, there is a benefit to breaking the infectivity of the effector molecules before disseminating them around the organism. I usually describe this by analogy to antiviral software: If you are worried about viruses infecting your computer, you will buy an antiviral software package that carries (i) a database of information about viruses (computer viruses in that case), (ii) a series of routines to establish which files are infected, and (iii) a series of remedies which either correct or delete the infected filed. The virus database that is part of this package doesn't need to have complete sequences for each virus, and indeed it would be a mistake for the antiviral software company to distribute such a database, as some of the components from the database might end up initiating infections. By taking from each virus only a set of relatively short signature sequences, it becomes possible to distribute identifying information without distributing the potential for infectivity. Breaking the double stranded RNA into 21–25 nt segments may serve the same role in cellular responses to unwanted RNA.

Dissemination of immune effector information is another feature of both classical and RNA-based immune mechanisms. For classical immunity this involves hitch-hiking with the blood circulation that permeates the body, as well as some very highly choreographed lymphocyte migration processes. For RNA-based immunity, the mechanisms of information dissemination are still being unveiled. Results demonstrating a concerted protein-based machinery that mediates dissemination of the RNAi response in *C. elegans* [e.g., 107] are

certainly exciting; understanding this machinery will be of great interest in designing and planning applications of RNAi.

RNAi, like any cellular mechanism, requires use of energy and metabolic resources. Balancing those resources with the current needs of the organism, and focusing the resources available for this purpose on the most pressing dangers, are essential for the system to fulfill its worth. For classic immunity, there are mechanisms that manage the population of effector molecules involved in surveillance (T and B cell repertoire), both by subtracting out specificities that are not engaging targets and by amplifying specificities that engage their targets. One expects, perhaps, to find similar overall management of specificities that guide the RNAi machinery. An enticing example of such management comes from the involvement of RNA-directed RNA polymerases in the silencing process for plants, worms, and some single-celled organisms (See Figure 11). First characterized in plant systems in the 1970s, cellular enzymes that can copy RNA to RNA [e.g., 108] had little place in the central dogma of molecular biology (DNA makes RNA makes Protein). Considerable doubt regarding the source of such enzymes inhibited research until they were purified and shown to be encoded by the cellular genome [109–110] and subsequently shown to play key roles in RNAi in *Neurospora*, worms, and plants [111–115]. One of the striking aspects of RdRP-based trigger amplification that has been described is that amplification only occurs when a target has been engaged. The consequences of this guidance mechanism [116–122] are (i) that amplification of the effector signal is limited to cases in which there is a real target, and (ii) that the spectrum of RNA silencing triggers can spread outside of the original area to encompass a broader segment of a target that has been recognized as foreign/unwanted. The RdRP-based amplification mechanism thus provides an example of honing the immune activity of the RNAi system to "clear and present" dangers.

The immune system analogy to RNA-based surveillance brings up a final question of how the system can remember prior challenges to provide optimal immunity. For the majority of RNA interference experiments done in *C. elegans*, the visible effect disappears after a generation or so [e.g., 40]. This is not always the case however, and there are instances in which gene-specific effects of RNAi can last for numerous generations [e.g., 123–124]. Similar long term effects have been studied in plant systems [e.g., 125]. Such effects would not be expected from the simple model in Figure 9. Instead, a current model (see Figure 12) is that the initial interaction between effector and target sequences might have a combination of short term consequences (e.g. inhibition of translation and degradation of the target mRNA), medium term consequences (such as production of additional small RNA effectors complementary to the target) and long term consequences (including changes in the physical conformation [chromatin context; 46,47] of the cellular DNA that encodes the target transcript). This variety of responses to a similar initial interaction event is in many ways analogous to the classic immune system, where an initial target recognition interaction can lead to a plethora of downstream consequences. In each case, the initial interaction complexes

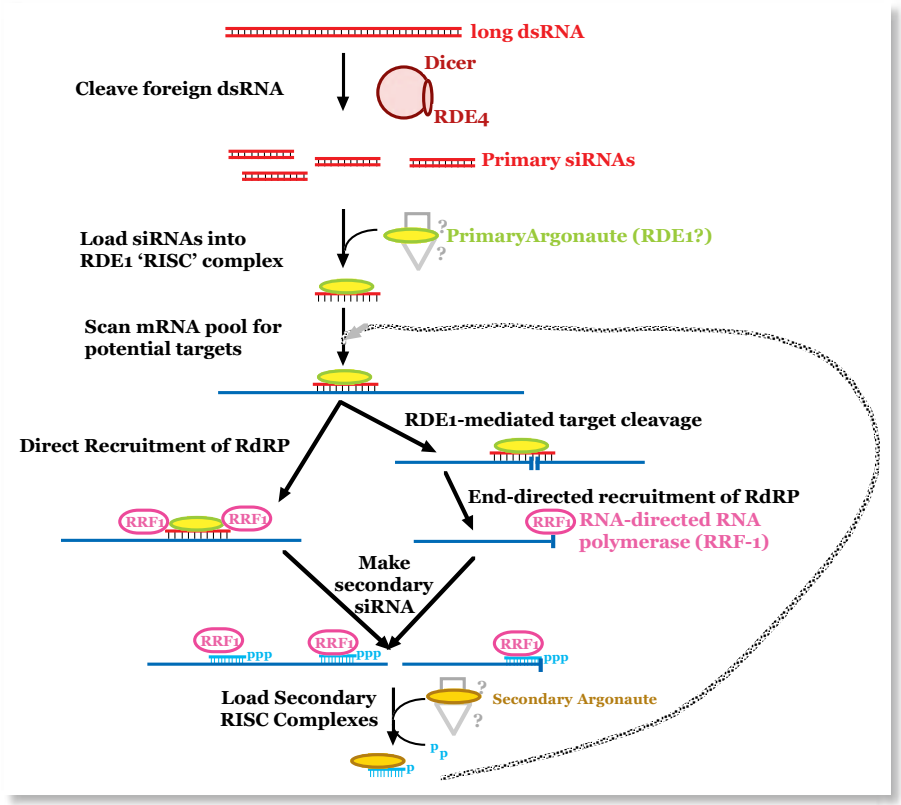

Figure 11. A model for amplified RNA interference in *C. elegans* somatic tissue. Based on discussion and references in the text, long dsRNA introduced into cells is initially attacked by a complex of a nuclease (Dicer) and a recognition component (RDE4) that "dice" the long dsRNA into short fragments. Loading of these fragments into a second protein complex results in a silencing complex that can scan the message population of the cell for matching sequences. These are then subject to two different consequences: cleavage (which should inactivate the message) and/or synthesis of short complementary RNAs [116–122]. The short complementary RNAs can join their own effector complexes (possibly including a different Argonaute family member, see [143]), resulting in a target-dependent amplification of the foreign dsRNA response. See text and references for further discussion of this proposed mechanism.

(RISC-mediated nucleic acid hybridization in the case or RNAi, antibody: antigen or T-Cell-Receptor::antigen in the case of the classic immune system) appear capable of recruiting a diversity of suppressive mechanisms based on the circumstances, with the duration of any given response (and subsequent memory) depending on a balance between longer and shorter term consequences.

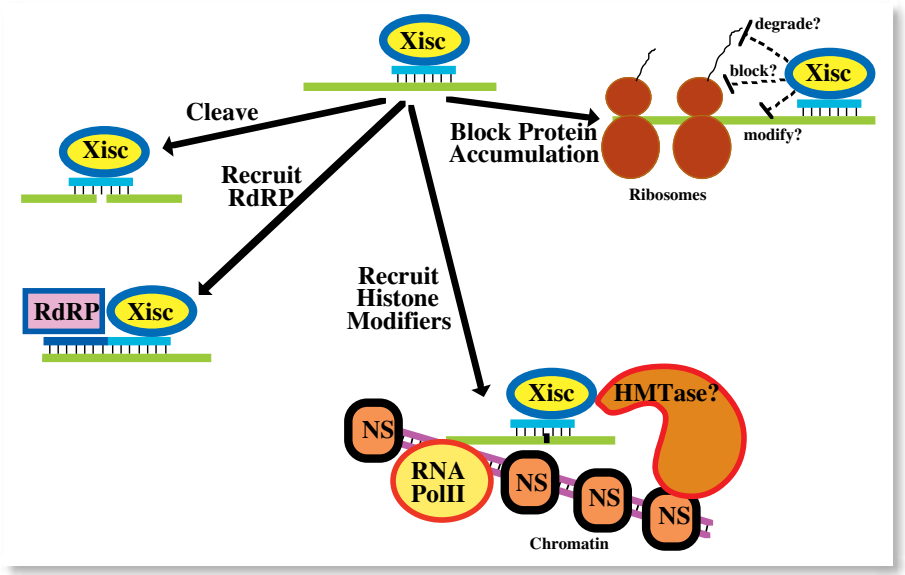

Figure 12. A model for multi-modal gene silencing as a result of siRNA effector recognition of RNA transcripts. A generic Argonaute:siRNA:target ternary complex is shown at the top, giving rise in principal to several different complexes in which silencing factors have been recruited. Left, top, cleavage of target transcript by Argonaute-like component or a recruited ally. Left, middle, recruitment of an RNA-directed RNA polymerase that might synthesize complementary RNA either primed by the initial siRNA or (as appears to be the case in *C. elegans*) with de-novo initiation. Bottom, a rough schematic diagram of tethered chromatin modification components acting on nearby nucleosomes and/or other DNA-associated factors (drawn here as a silencing HMT-ase = "Histone methyltransferase" [see 145] although numerous other epigenetic modifying activities could function equivalently). Note that this process would likely occur on a nascent RNA transcript still associated physically with the DNA template [144]. Right, recruitment of factor(s) that might block translation of the message [e.g., 48].

GOING FORWARD: PUZZLES AND CHALLENGES

RNAi is an extremely active field of current investigation and will certainly remain so for some time. Many of the central questions relate to basic mechanisms; many others relate to potential applications. From the perspective of understanding RNAi as a potential immune-type surveillance mechanism, several questions currently occupy the forefront (I have cobbled together a list in Figure 13). One question concerns possible roles for RNAi as an antiviral response outside of the plant kingdom. Several recent studies in invertebrate animals (worms and flies) rather clearly show the capability of RNAi to function in surveillance against viruses (and other selfish information such as transposons) in simple animals [e.g., 85,126–128]. That the issue has not yet been resolved for higher animals (mammals) could conceivably reflect the complexity of teasing apart specific and non-specific responses of mammals

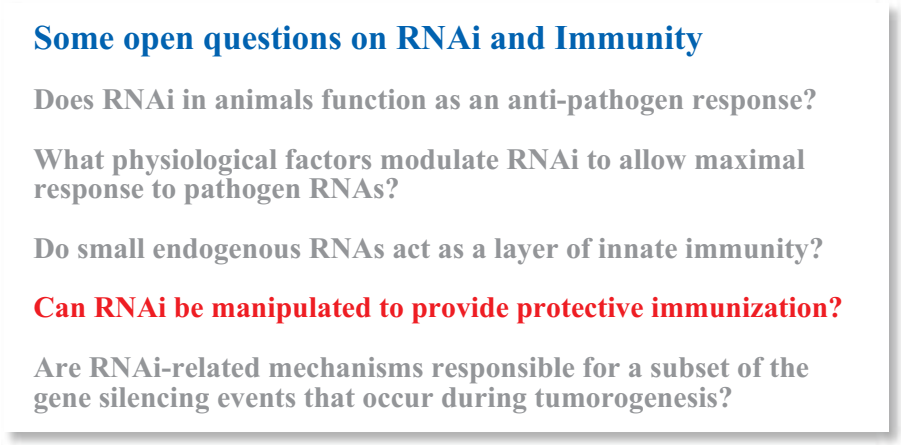

Figure 13. Some open questions on RNAi and immunity.

to dsRNA. Alternatively, it is certainly conceivable at this point that the virus-protective role of RNAi has been lost in mammals.

One exciting development over the last several years has been the appearance in the literature of detailed structures of components parts of the RNA interference machinery [e.g., 129,130]. These structures have, both individually and in aggregate, led to an understanding of aspects of the mechanism would have only been dreams about during the early phases of the analysis of the system. With the emerging structural wisdom come a large number of thermodynamic and kinetic questions. For the less technically inclined reader, these challenge us to understand the contributions of energy and equilibrium to the natural system and to add the dimension of time to the static pictures such as those in Figures 9 and 11. Already it is clear that kinetic competition between different potential effectors at each stage of the RNAi mechanism is a key determinant of how the RNA-based surveillance system is used [e.g., 131,132]. Likewise, kinetic competition between the RNAi machinery and other protein: RNA interactions (RNA synthesis and processing machinery, RNA storage and turnover machinery, and the translation machinery) will undoubtedly determine the spectrum of RNAi events that can actually occur during the life of a cell [e.g., 133].

At the same time as detailed biochemical and structural studies are likely to illuminate the forefront of RNAi, there is still much to be learned from genetic analysis. The original screens of Tabara *et al.* [85] found just two *C. elegans* genes with the idealized property that they eliminated almost all RNA interference with little or no effect on the organism. Similarly, limited sets of comparable genes (although different individual components) were identified in the early genetic screens of plant and fungal systems [e.g., 93,94]. Vertebrate cells that lack the major Argonaute component involved in dsRNA-based surveillance are intriguingly alive (and capable of growth in a Petri dish) but incapable of forming a viable organism [134].

Correspondingly, some mutants in other systems that may have superficially appeared specific to the dsRNA response also exhibit intriguing variations in growth and/or physiology even in the absence of known pathogenic challenges [e.g., 135,136]. In addition to these observations, several biological forces which were limiting the original genetic screens are now clear. In some cases, the failure to recover mutants affecting a given stage in the process reflected a degree of genomic redundancy, with several different gene products each sufficient (at least partially) to execute a single reaction step [e.g., 85]. Conversely, some RNAi components were not identified in the early screens due to their shared involvement in RNAi-related (but distinct) processes which use similar molecular machineries and which are essential for organismal viability. In addition to the well characterized micro RNA regulatory system [128,137], the portfolio of RNAi-related processes will almost certainly include surveillance and regulatory roles within cells which we have yet to understand [e.g., 128]. As the expanding toolkit for analyzing essential and redundant genes in genetic model systems is applied, we should be able to open more than a few doors toward illumination of both the natural roles of RNAi and of numerous yet-to-be-elucidated cellular regulatory and surveillance functions.

RNA INTERFERENCE AS A TOOL IN MEDICINE?

A question that has generated considerable excitement beyond the research lab is whether effector dsRNAs might be used as a direct intervention to treat human disease. Indirect applications of RNAi in medicine have certainly jumped forward: RNAi takes its place among many different tools to understand gene regulation, assign functions to individual genes, and facilitate the discovery of potential therapeutic targets in disease systems.

Will direct administration of interfering RNA be a useful clinical tool? If a person has a virus infection, why not use double stranded RNA corresponding to that viral sequence as a drug to treat the person? If a person has a tumor, why not take a gene that's essential for that tumor and administer double stranded RNA corresponding to that gene to shut down growth of that tumor? If a person has a disease caused by an altered or out-of-control gene, why not try double stranded RNA corresponding to that gene as a potential therapeutic? There are many challenges and many conceivable benefits to this approach. There are scores of potential applications, all of which will require negotiating the thicket of delivery, safety, and efficacy in the complex circumstance of a genetically diverse target population and with the need to understand and anticipate host (and in some cases pathogen) responses to the specific dsRNA. Maybe the time frame in testing these approaches will be years, maybe tens of years, and maybe more. With all of the trepidation and caution that goes into such an enterprise, I still look forward to seeing research in this area progress as a future endeavor in both the public and the private sector.

I expect that there will be additional areas (beyond the gene discovery and therapeutic RNAi applications discussed above) in which understanding of RNA-triggered gene silencing will provide therapeutic opportunities and augment to our capacity to mitigate disease. Any potent and specific biological process (even if it is generally beneficial to the organism) comes with consequences to the organism if abnormalities in specificity or regulation occur. Aberrations in genetic silencing (both positive and negative) are certainly a major component of many human diseases, including most prominently cancer. Intensive investigations of dysregulation in cancer and other disease have turned up cases of defects in virtually every known cellular regulatory pathway. Regulation by small RNAs has rapidly joined this group [e.g., 139,140], with currently available data likely accounting for only a small fraction of such effects. As the potential contributions of RNA-triggered genetic silencing processes to both disease and the human response to disease continue to be characterized, it is conceivable that there will be clear cases in which manipulation of the RNAi machinery itself, either in a global manner or in a small subset of cells or effector functions, will become an attractive therapeutic strategy. As such situations arise, the availability of therapeutic interventions to manipulate aspects of the RNAi machinery such as small molecule drugs [e.g., 141] and biologically-based modulatory strategies [e.g., using viral anti-silencing components] will certainly provide worthy leads for potential treatment.

SCIENCE DOESN'T GROW ON TREES, EVEN IN SANTA CLARA COUNTY...

I want to finish with a few thanks. I have been fortunate to be associated with a family, a group of friends, a set of co-workers, and a number of institutions for which scientific inquiry and humanity have been equally highly valued. This has made it a joy to do science.

Since this article focuses most directly on experiments from the 1990s on the structural trigger for RNAi, I want to first specifically acknowledge the members of my lab and some of our collaborators that were directly involved in this work. The crew in my lab that were involved most directly in this particular effort were SiQun Xu, Mary Montgomery, Steve Kostas, Lisa Timmons, Susan White-Harrison, Jamie Fleenor, and Susan Parrish. Collaborations with Craig Mello and his group, particularly Sam Driver and Hiroaki Tabara likewise drove of the effort in wonderful ways, as did collaborations with Natasha Caplen and Rick Morgan at NIH, Farhad Imani at Johns Hopkins, and Titia Sijen, Femke Simmer, Karen Thijsen, and Ronald Plasterk at Utrecht. I hope you realize that even this rather substantial scientific consortium was just a piece of a very large puzzle that involves also many other scientists and groups around the world.

Two institutions deserve particular attribution for any contributions from myself and my lab. The Carnegie Institution of Washington's Department of Embryology is where we did our work on the structural characterization of

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For anybody to make their way in the world, there need to be inputs and contributions... and a lot of influences. When I sat down to put a few names down of people that at one point or another have had a positive influence, Figure 14 emerged. I apologize in advance for any inadvertent omissions (and numerous spelling errors)... you know who you are.

Figure 14. A few of the people and groups that the author would like to acknowledge for their help, support, encouragement. The list is in computationally randomized order with a few omissions (apologies) and misspellings (apologies).

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Portrait photo of Dr. Fire by photographer Linda Cicero.