RNAi minilecture and Using Genetics to Explore Complex Biological Processes

2 American ‘Worm People’ Win Nobel for RNA Work
New York Times Oct. 2, 2006    The 2006 Nobel Prize in Physiology or Medicine was awarded to two American researchers, Andrew Z. Fire and Craig C. Mello, for a far-reaching discovery about how genes are controlled within living cells.

The discovery was made in 1998, only eight years earlier. .......The finding by Drs. Fire and Mello made sense of a series of puzzling results obtained mostly by plant biologists, including some who were trying to change the color of petunias. By clarifying what was happening, they discovered an unexpected system of gene regulation in living cells and began an explosive phase of research in a field known variously as RNA interference or gene silencing. This natural method of switching genes off has turned out to be a superb research tool, allowing scientists to understand the role of new genes by suppressing them. The method may also lead to a new class of drugs that switch off unwanted processes in disease.

Michael Probst/The Associated Press
Craig C. Mello, right, and Andrew Z. Fire at an awards ceremony in Germany in March.
IN the BEGINNING was RNA

Timeline for the Universe suggesting the early existence of an RNA world of living systems

What are the “Traditional” Roles of RNA in the cell?
YOU DON’T KNOW WHAT YOU DON’T KNOW: NEW ROLE FOR RNA DISCOVERED IN THE PAST COUPLE OF YEARS:
Scientific discovery by serendipity:
The plant thread of the story begins with the search for a more purple flower

The quest for purpler petunias

- Plant biotechnologists strategy was to try to boost the activity of an enzyme involved in the production of anthrocyanin pigments
- The researchers hooked up the gene to a powerful promoter sequence and introduced this artificial construct into their petunias
- The investigators expected deep purple flowers from a high level of transcription of the transgene
- Instead of being deep purple, many of the flowers grew up virgin white or variegated
- In the white or variegated flowers, not only was the transgene not activated, but the endogenous anthrocyanin genes had been inactivated
- the white phenotype could be passed onto the next generation -- but some flowers reverted to purple
- Was this phenomenon controlled by some sort of unstable nucleic acid?
The worm thread of the story: when controls don’t behave properly

Older naïve idea:: antisense technology*

Unexpected results from controls suggested that “antisense” techniques weren’t functioning via the expected mechanism:

• sense RNA also worked to abrogate gene function
• double-stranded RNA worked 10 times better than sense or antisense RNA

• The notion that you could use an RNA complementary to the mRNA from a specific gene to abrogate gene function
RNA interference
-- gene silencing by double-stranded RNA

1. The central dogma

Our genome operates by sending information from double-stranded DNA in the nucleus, via single-stranded mRNA, to guide the synthesis of proteins in the cytoplasm.

2. The experiment

RNA carrying the code for a muscle protein is injected into the worm C. elegans. Single-stranded RNA has no effect. But when double-stranded RNA is injected, the worm starts twitching in a similar way to worms carrying a defective gene for the muscle protein.

- Sense RNA
- Antisense RNA
- Double-stranded RNA

Parent

Offspring

- No effect
- No effect
- Twitching

3. The RNAi mechanism

RNA interference (RNAi) is an important biological mechanism in the regulation of gene expression.

- Double-stranded RNA (dsRNA) binds to the protein Dicer...
- ... which cleaves dsRNA into smaller fragments.
- One of the RNA strands is loaded into a RISC complex...
- ... and links the complex to the mRNA strand by basepairing.
- mRNA is cleaved and destroyed. No protein can be synthesized.

What Fire and Mello established
Fire and Mello established that

• That double-stranded RNA was the “active gene knockout agent” and that previous results showing effects of single-stranded antisense (or sense) RNA were due to double-stranded RNA that contaminated the preps
• Double-stranded RNA interfered specifically with the function of the sequences that coded for the RNA
These and other investigations (with funny outcomes) in other organisms converged on an ancient RNA silencing system that is conserved in fungi, plants and animals.

**RNAi has roles in:**

- normal developmental events that are controlled by micro RNAs (miRNAs)
- an ancient “immune system” that protects cells from foreign (rougue) and/or aberrant nucleic acids
Gagging order: using dsRNA, specific genes can be silenced

HUH? WHAT?
How does it work?
What triggers it?
How have molecular biologists made use of it?

How do we know about dicer?

How did we get from the initial observations to the detail on the next page?
How do we know this?
RNA silencing involves molecular machines

Figure 2 Dicer and RISC (RNA-induced silencing complex). a, RNAi is initiated by the Dicer enzyme (two Dicer molecules with five domains each are shown), which processes double-stranded RNA into 22-nucleotide small interfering RNAs36. Based upon the known mechanisms for the RNase III family of enzymes, Dicer is thought to work as a dimeric enzyme. Cleavage into precisely sized fragments is determined by the fact that one of the active sites in each Dicer protein is defective (indicated by an asterisk), shifting the periodicity of cleavage from 9–11 nucleotides for bacterial RNase III to 22 nucleotides for Dicer family members40. The siRNAs are incorporated into a multicomponent nuclease, RISC (green). Recent reports suggest that RISC must be activated from a latent form, containing a double-stranded siRNA to an active form, RISC*, by unwinding of siRNAs41. RISC* then uses the unwound siRNA as a guide to substrate selection31. b, Diagrammatic representation of Dicer binding and cleaving dsRNA (for clarity, not all the Dicer domains are shown, and the two separate Dicer molecules are coloured differently). Deviations from the consensus RNase III active site in the second RNase III domain inactivate the central catalytic sites, resulting in cleavage at 22-nucleotide intervals
How do we know this?
and this?

how would you even get a foothold?
Mello and Fire used a forward genetics approach (involving random mutagenesis) to discover the genes that coded for proteins that were part of the RNAi system in worms.

**HOW did they do this?**
A closer look at *Caenorhabditis elegans*

- C. elegans was plucked out of obscurity a few decades ago and now we probably know more about this than we know about any other metazoan -- excepting *Drosophila*

- IN the 1960’s Sidney Brenner pick C. elegans as a great candidate for a model system that could be used to explore the genetic control of nervous system development

- Eventually became clear that this worm could be used to study the genetic control of many developmental processes
What we know about C. elegans:
1. Adult hermaphrodite has 959 somatic cells and the male has 1031
2. The complete cell lineage has been determined and the fate of each cell is known (since the animal is transparent, cell divisions can be observed in the living organism)
3. There are 302 neurons in the adult hermaphrodite and a complete wiring diagram of the adult nervous system has been generated (all the connections of the nerves)
4. The entire genome of the worm (97 Mb) has been sequenced, that is, the complete DNA sequence has been determined
5. The worm’s genome contains 19,099 genes
   • 27% of the genome is coding sequence
   • 26% is in introns
   • 32% of worm sequences are similar to human sequences

Using a model system and a genetic approach to studying a complex biological problem

• How is the nervous system specified
• How does a developing embryo specify its head and its tail
  • How does sex determination work?
• How does a developing embryo assemble a complex array of tissues and put them in the correct location and orientation
• How does DNA replication occur?
• How do cells control the cell cycle
Intro to second worm lab:

How does this type of sex determination “work” at the molecular level?

| XX | X:A ratio | Low | High | Low | High | Low | High | Gene names in middle of diagram |
|----|-----------|-----|------|-----|------|-----|------| High = high levels of gene expression |
|    |           |     |      |     |      |     |      | Low = low levels |

Male

Hermaphrodite
How to identify genes that are important for assessing $X:A$ ratio and translating this signal into male or hermaphrodite development?

- the mutants
- explanations for the mutant phenotypes?
More on RNAi

Science 296: 1263 A model for the molecular steps in RNA silencing RNAi animation featuring species differences in the RNAi specifics:
http://imagenex.com/rnai_anim.php

More acronyms: RdRP = RNA-dependent RNA polymerase
RNA-induced silencing complex = RISC
siRNA = small interfering RNA

What makes a ssRNA aberrant?
How does it work? Although mechanisms of gene silencing are far from completely understood, the working hypothesis goes like this: the initial trigger is the presence in the host's cells of an aberrant RNA. This could be a double-stranded RNA, a shortened RNA that lacks its 'cap' or 'tail', or a conventional RNA that is present in unusually large quantities. The host organism's response is to call on enzymes that slice and dice the offending RNA into pieces around 25 nucleotides long. At some stage — either before or after the formation of these fragments — the rogue RNA is copied many times over, to amplify the alarm signal. The fragments then spread throughout the host. Antisense strands, complementary to the target mRNA, bind to the target and prompt other enzymes to disable it