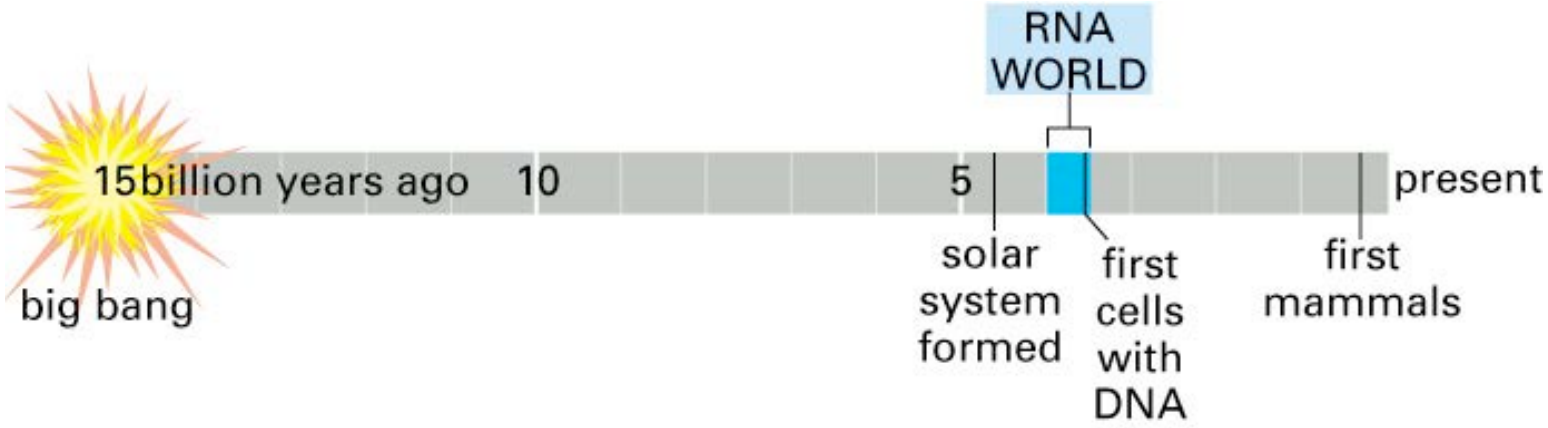
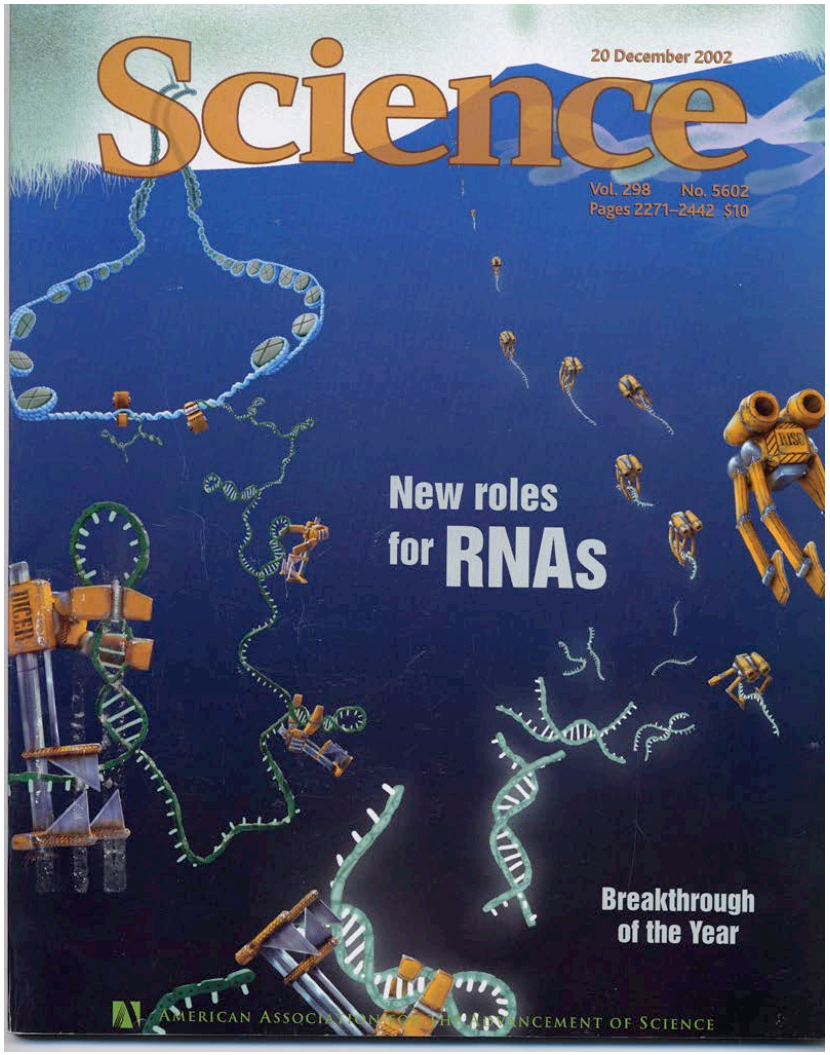
IN the BEGINNING was RNA

Why do biologists think this?



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 ROLES FOR RNA DISCOVERED – even in **YOUR** lifetime!*

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.

Craig C. Mello, right, and Andrew Z. Fire at an awards ceremony in Germany in March.

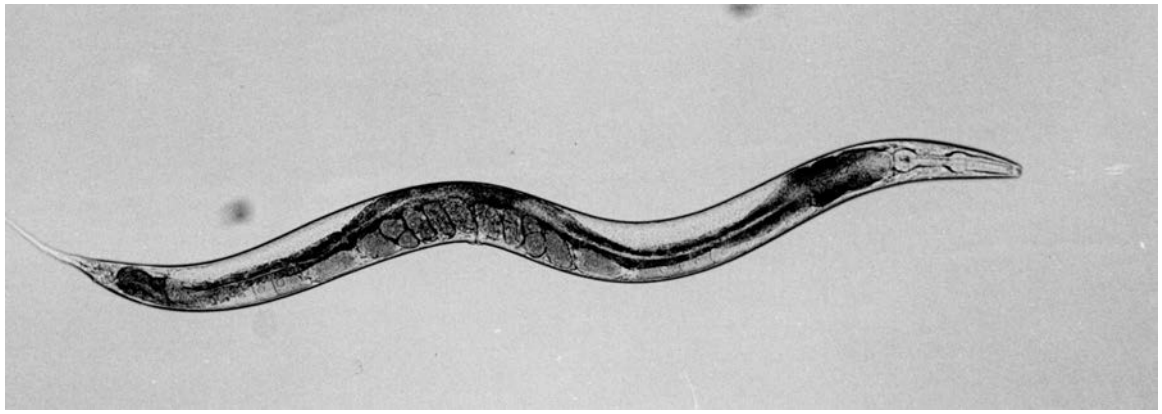
The discovery was made in 1998, only eight years earlier. *.....The finding by Drs. Fire and Mello made sense of a series of puzzling results obtained mostly by plant biologists, including some who were trying to change the color of petunias.* By clarifying what was happening, they discovered an unexpected 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.

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 completely 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 phenonmenon controlled by some sort of unstable nucleic acid?

COVER The phenomenon now known as RNA interference (RNAi) was first uncovered as a sequence-specific gene silencing response provoked by the introduction of exogenous multicopy transgenes into petunia. This resulted in flowers with two-toned color patterns, as shown here. The crystal structure of Argonaute, a signature component of the RNAi effector complex, is shown with its PAZ domain in blue and PIWI domain in purple. See page 1409, 1434, and 1437. [Petunia image, R. Jorgensen; ribbon diagram, L. Joshua-Tor]

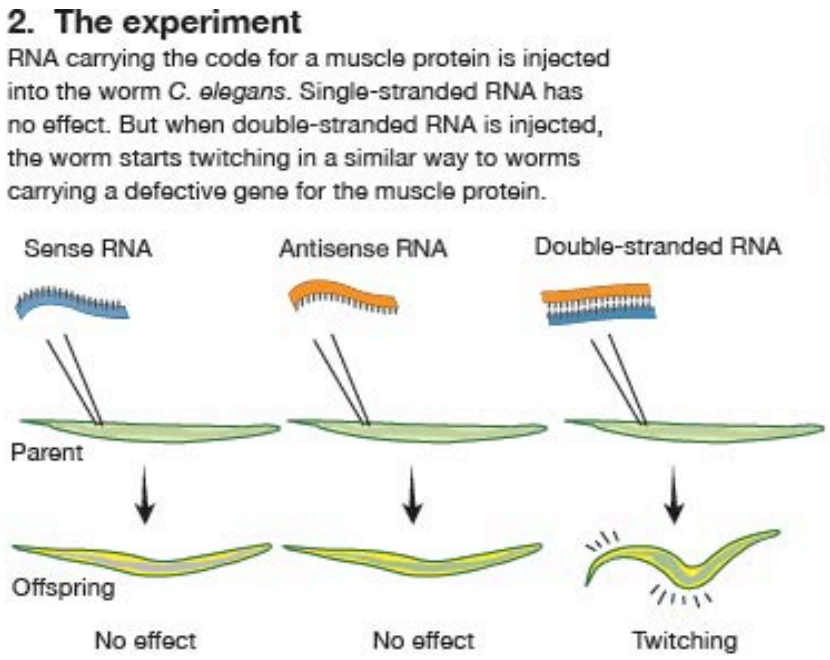


The worm thread of the story: when controls don't behave properly

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*



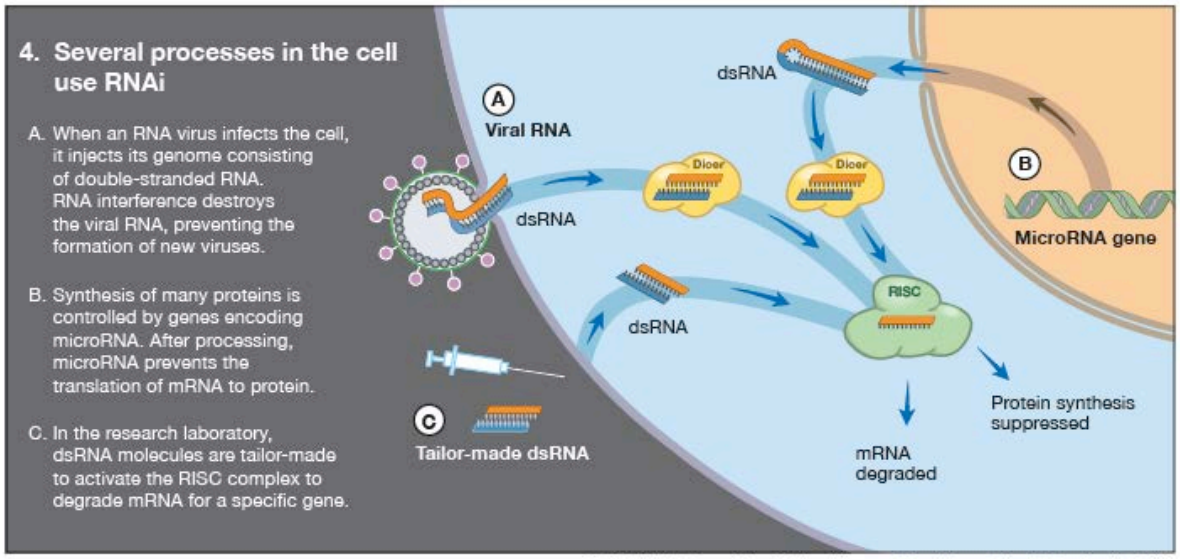
Fire and Mello established *that double-stranded RNA was the "active gene knockout agent" and that previous results showing effects of single-stranded antisense (or sense) RNA were due to double-stranded RNA that contaminated the preps*

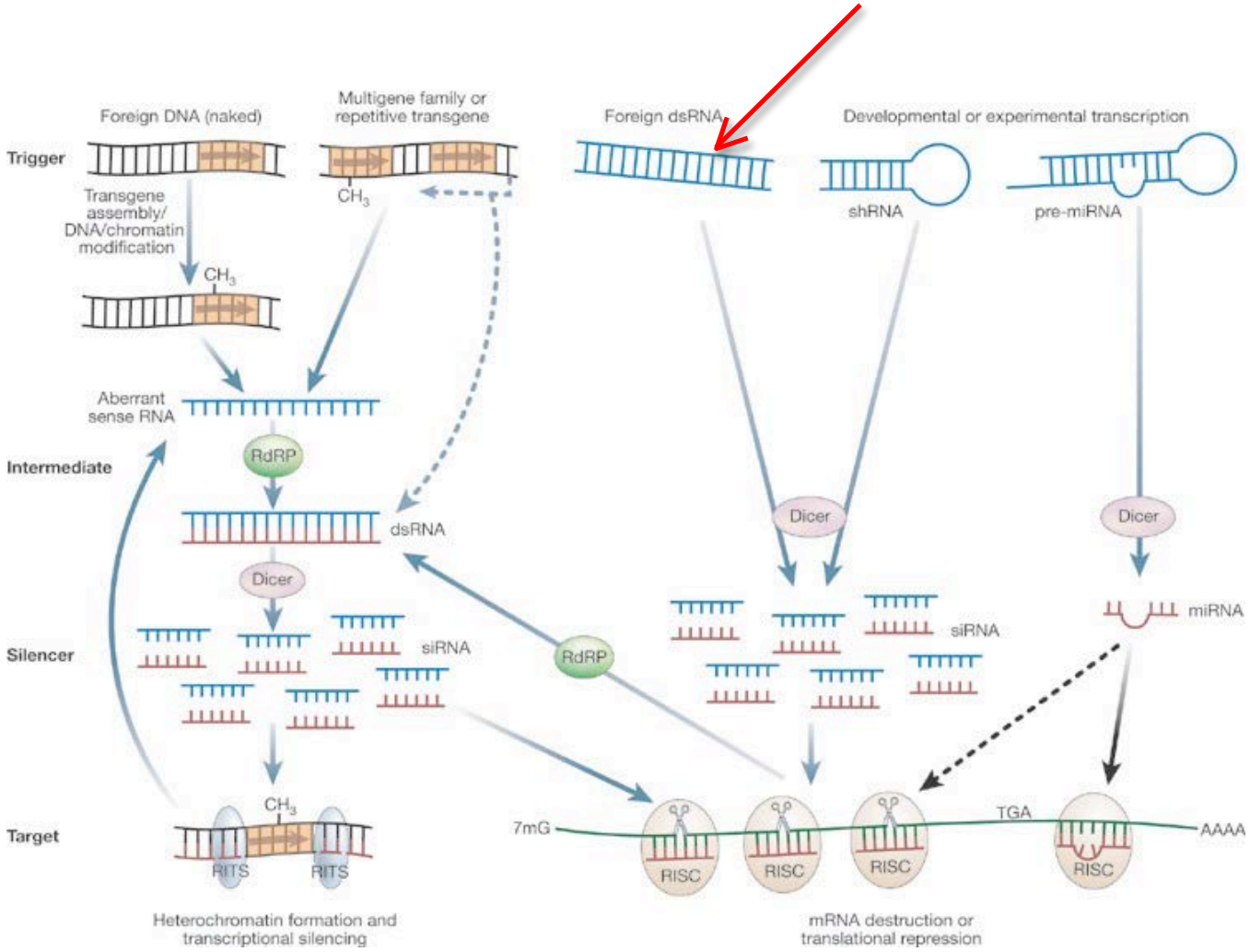
- *They and others observed that sequences matching a gene's promoter or its introns* ***were not effective*** *in inducing RNA interference*
- *Steady-state levels of mature cytoplasmic mRNAs were significantly reduced suggesting that RNAi operated via a post-transcriptonal targeting of the mature mRNA*

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 (siRNAs)





Model depicting distinct roles for dsRNA in a network of interacting silencing pathways. Legend to figure is on the next page

CH_3, modified DNA or chromatin; AAAA, poly-adenosine tail; TGA, translation termination codon ⇦ copied directly from legend in the paper!

Legend to figure on previous page; Model depicting distinct roles for dsRNA in a network of interacting silencing pathways.

- *In some cases dsRNA functions as the initial stimulus (or trigger), for example when foreign dsRNA is introduced experimentally.*
- In other cases dsRNA acts as an intermediate, for example when 'aberrant' mRNAs are copied by cellular RdRP.
- Transcription can produce dsRNA by readthrough from adjacent transcripts, as may occur for repetitive gene families or high-copy arrays (blue dashed arrows).
- Alternatively, transcription may be triggered experimentally or developmentally, for example in the expression of short hairpin **(shRNA)** genes and endogenous hairpin **(miRNA)** genes.
- **siRNAs**, the small (~23 nt) RNA products of the Dicer- mediated dsRNA processing reaction guide distinct protein complexes to their targets.

These silencing complexes include:

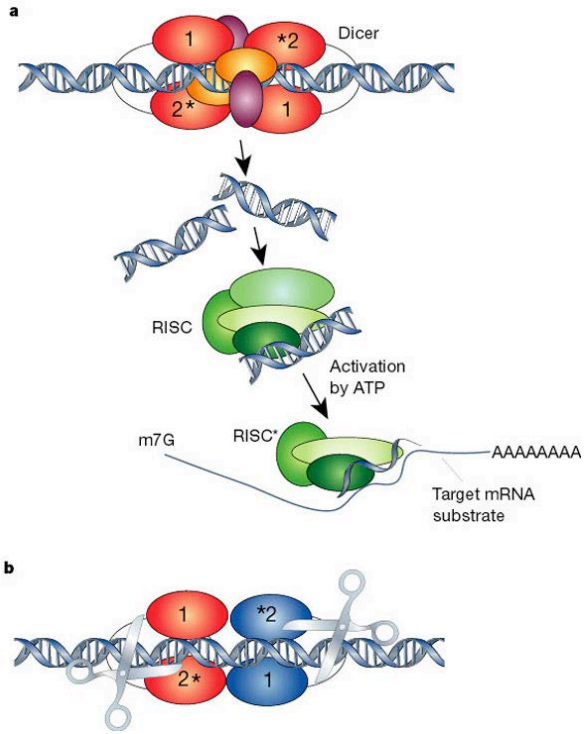
- the **RNA-induced silencing complex (RISC)**, which is implicated in mRNA destruction and translational repression, and
- the **RNA-induced transcriptional silencing complex (RITS)**, which is implicated in chromatin silencing.

Sequence mismatches between a miRNA and its target mRNA lead to translational repression (black solid arrow), whereas near perfect complementarity results in mRNA destruction (black dashed arrow). Feedback cycles permit an amplification and long-term maintenance of silencing.

How do we know this?

RNAi animation featuring species differences in the RNAi specifics:
http://imgenex.com/rnai_anim.php
Check out this RNAi animation:
http://www.nature.com/focus/rnai/animations/index.html

RNA silencing involves molecular machines



Nature 418: 244

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 RNAs. 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 members. 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 siRNAs. RISC* then uses the unwound siRNA as a guide to substrate selection.

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

Mello and Fire used a traditional forward genetics strategy to identify the molecular components of the worm RNAi pathway

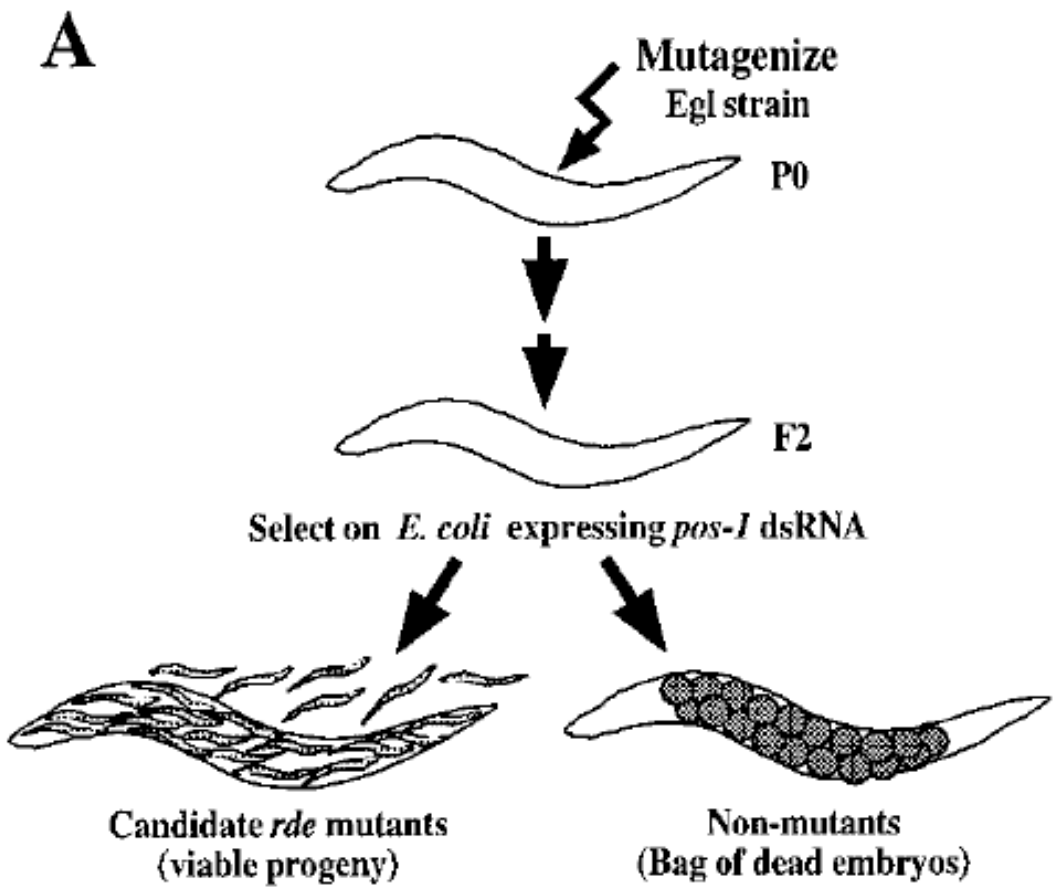
Cell, Vol. 99, 123–132, October 15, 1999

The *rde-1* Gene, RNA Interference, and Transposon Silencing in *C. elegans*

Summary

Double-stranded (ds) RNA can induce sequence-specific inhibition of gene function in several organisms. However, both the mechanism and the physiological role of the interference process remain mysterious. In order to study the interference process, we have selected *C. elegans* mutants resistant to dsRNA-mediated interference (RNAi). Two loci, *rde-1* and *rde-4*, are defined by mutants strongly resistant to RNAi but with no obvious defects in growth or development. We show that *rde-1* is a member of the *piwi/sting/argonaute/zwille/eIF2C* gene family conserved from plants to vertebrates. Interestingly, several, but not all, RNAi-deficient strains exhibit mobilization of the endogenous transposons. We discuss implications for the mechanism of RNAi and the possibility that one natural function of RNAi is transposon silencing.

Here, we report the identification of *R*NA interference-*de*ficient (*rde*) mutants in *C. elegans*. Mutations that greatly reduce or abolish RNA interference arise at frequencies expected for simple recessive loss-of-function mutations. *rde-1* and *rde-4* mutants appear to completely lack an interference response to several dsRNAs tested but are, nevertheless, healthy and viable under laboratory conditions, suggesting that at least some steps in the interference process are nonessential. We show that *rde-1* encodes a novel member of an ancient gene family that includes plant, *C. elegans*, *Drosophila*, and vertebrate homologs. Genetic studies have implicated several *rde-1* family members in germline maintenance and development (Bohmert et al., 1998; Cox et



Identification of RNAi deficient mutant strains

HIDING IN PLAIN SITE

The argonaute family of endonucleases was well known before Fire and Mello discovered the rde genes, but, its biological role was not known

This family was first linked to RNAi through these forward genetic studies in Caenorhabditis elegans, which identified rde-1 as a gene essential for silencing.

Cell. 1999 Oct 15;99(2):123-32.

The rde-1 gene, RNA interference, and transposon silencing in C. elegans.

Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, Mello CC.

Abstract

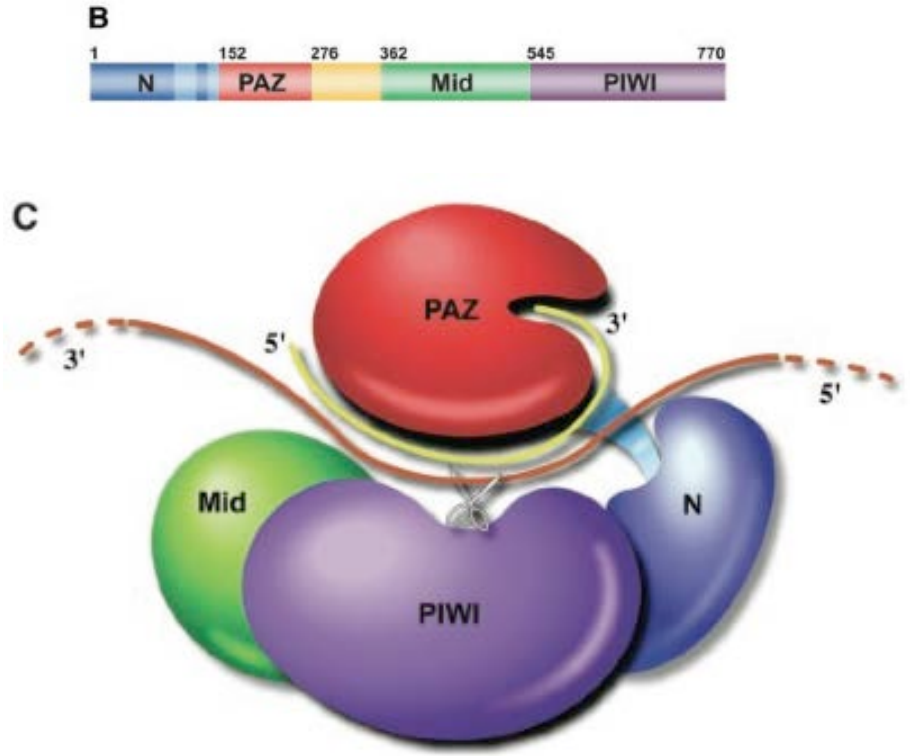
Double-stranded (ds) RNA can induce sequence-specific inhibition of gene function in several organisms. However, both the mechanism and the physiological role of the interference process remain mysterious. In order to study the interference process, we have selected C. elegans mutants resistant to dsRNA-mediated interference (RNAi). Two loci, rde-1 and rde-4, are defined by mutants strongly resistant to RNAi but with no obvious defects in growth or development. ***We show that rde-1 is a member of the piwi/sting/argonaute/zwille/eIF2C gene family conserved from plants to vertebrates.*** Interestingly, several, but not all, RNAi-deficient strains exhibit mobilization of the endogenous transposons. We discuss implications for the mechanism of RNAi and the possibility that one natural function of RNAi is transposon silencing.

Crystal Structure of Argonaute and Its Implications for RISC Slicer Activity

Science 305:1437 9/3/04

(B) Schematic diagram of the domain borders.

(C) Schematic depiction of the model for siRNA-guided mRNA cleavage. The siRNA (yellow) binds with its 3' end in the PAZ cleft and the 5' is predicted to bind near the other end of the cleft. The mRNA (brown) comes in between the N-terminal and PAZ domains and out between the PAZ and middle domain. The active site in the PIWI domain (shown as scissors) cleaves the mRNA opposite the middle of the siRNA guide.

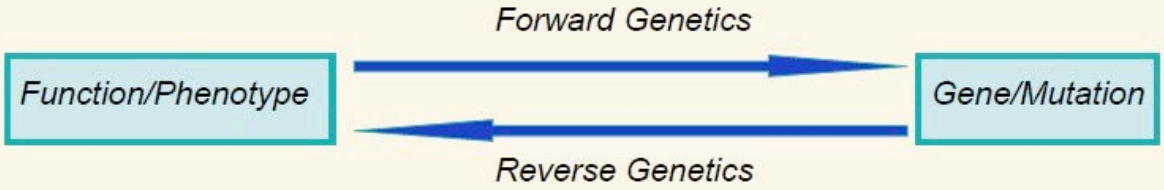


As laboratory tool, the RNAi system is useful for reverse genetics by targeted gene knockout

What is the genetic basis of a particular phenotype?
(How does one determine the function of a gene, or the identity of genes responsible for a trait?)

Forward Genetics:
Starts with a phenotype and moves towards the gene

Reverse Genetics:
Starts with a particular gene and assays the effect of its disruption



Reverse genetics*

Julie Ahringer, ed. §
The Gurdon Institute, University of Cambridge, Cambridge, CB2 1QN, UK

1. Introduction to reverse genetics in *C. elegans*

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

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

Introducing DS-RNA into an organism

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

1. **RNAi by injection:** dsRNA produced in vitro is injected into young adult hermaphrodites and their progeny scored for mutant phenotypes.

2. **RNAi by soaking:** worms are soaked in a high concentration dsRNA solution and then subsequently they or their progeny scored for phenotypes.

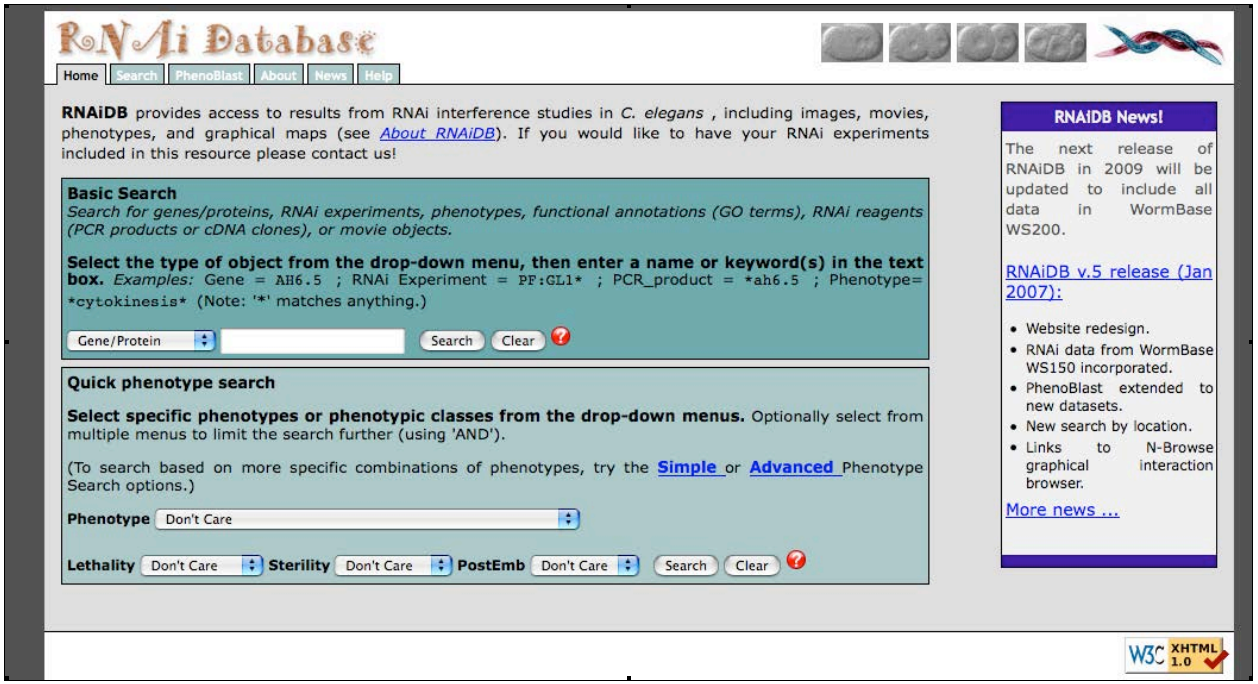
3. **RNAi by feeding:** *E. coli* producing the desired dsRNA are fed to worms and either they or their progeny are scored. Worms of any stage can be subjected to RNAi by feeding.

RNAi as a tool for targeted knockdown of gene expression
Libraries of clones for RNAi knockout

2.7. Where do I get RNAi clones?

There are currently two RNAi feeding libraries for *C. elegans*. One is from the Ahringer lab and has 16,757 clones, made by cloning gene-specific genomic fragments between two inverted T7 promoters (Fraser et al., 2000; Kamath et al., 2003). The inserts contain exons and introns and sizes vary from 500 bp to 2.5 kb. The other is from the Vidal lab and has 11,511 clones, made by the Gateway cloning of full-length open reading frame (ORF) cDNAs into a double T7 vector (Rual et al., 2004). Both libraries use the HT115 bacterial strain as a host for the plasmid RNAi clones; HT115 has IPTG inducible T7 polymerase and a disruption of the RNAse III gene (a dsRNAse), the latter marked with Tetracycline resistance (Timmons et al., 2001). There is some overlap between the two libraries; together they can target about 94% of *C. elegans* genes. Individual clones and whole libraries are available from Geneservice. The Vidal library is also available from Open Biosystems.

http://nematoda.bio.nyu.edu:8001/cgi-bin/index.cgi



HT115 = E. coli strain with:

- gene coding for the T7 virus polymerase under transcriptional control of the lac operator/repressor
- loss-of-function mutation in the gene coding for RNaseH (specific for dsRNA)

pL440 plasmid contructs:

- gene sequence is cloned into plasmid and flanked by inverted promoters specific for the T7 polymerase

HT115(DE3)/pL440(*bli-1*) – vector carries a segment of the ***bli-1 gene*** **HT115(DE3)/pL440(*dpy-11*)** - vector carries a segment of the ***dpy-11 gene***

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

***C. elegans* Strains:**

wildtype

***dpy-11(e224)V* – *dumpy* reference mutant strain**

***bli-1(e769)II* – *blister* reference mutant strain**

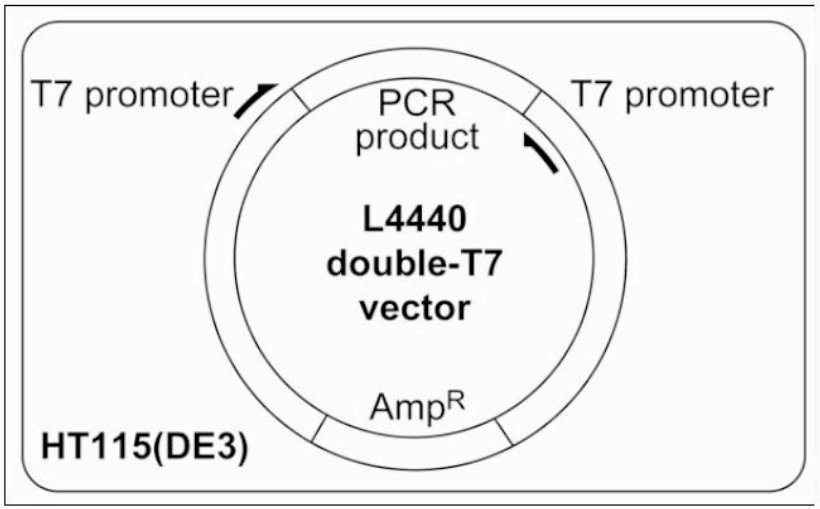


Figure 1

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

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

- T7 promoter = promoter sequences lifted from the genome of the T7 E. coli bacteriophage
- Transcription of the *C. elegans* sequences (PCR product in above map) can proceed using both strands as template because there are two T7 promoters flanking the sequence oriented on oppposite strands.
- The double-strand RNA is synthesized only when T7 RNA polymerase is produced in the cell. Its transcription is (artificially) under the control of the lac repressor protein which in turn is under allosteric control by the compound IPTG.

Vol 448|12 July 2007|doi:10.1038/nature05954 nature

ARTICLES

A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*

Georg Dietzl[1,2], Doris Chen[1], Frank Schnorrer[2], Kuan-Chung Su[1], Yulia Barinova[1], Michaela Fellner[1,2], Beate Gasser[1], Kaolin Kinsey[1,2], Silvia Oppel[1,2], Susanne Scheiblauer[1], Africa Couto[2], Vincent Marra[1], Krystyna Keleman[1,2] & Barry J. Dickson[1,2]

Forward genetic screens in model organisms have provided important insights into numerous aspects of development, physiology and pathology. With the availability of complete genome sequences and the introduction of RNA-mediated gene interference (RNAi), systematic reverse genetic screens are now also possible. Until now, such genome-wide RNAi screens have mostly been restricted to cultured cells and ubiquitous gene inactivation in *Caenorhabditis elegans*. This powerful approach has not yet been applied in a tissue-specific manner. Here we report the generation and validation of a genome-wide library of *Drosophila melanogaster* RNAi transgenes, enabling the conditional inactivation of gene function in specific tissues of the intact organism. Our RNAi transgenes consist of short gene fragments cloned as inverted repeats and expressed using the binary GAL4/UAS system. We generated 22,270 transgenic lines, covering 88% of the predicted protein-coding genes in the *Drosophila* genome. Molecular and phenotypic assays indicate that the majority of these transgenes are functional. Our transgenic RNAi library thus opens up the prospect of systematically analysing gene functions in any tissue and at any stage of the *Drosophila* lifespan.

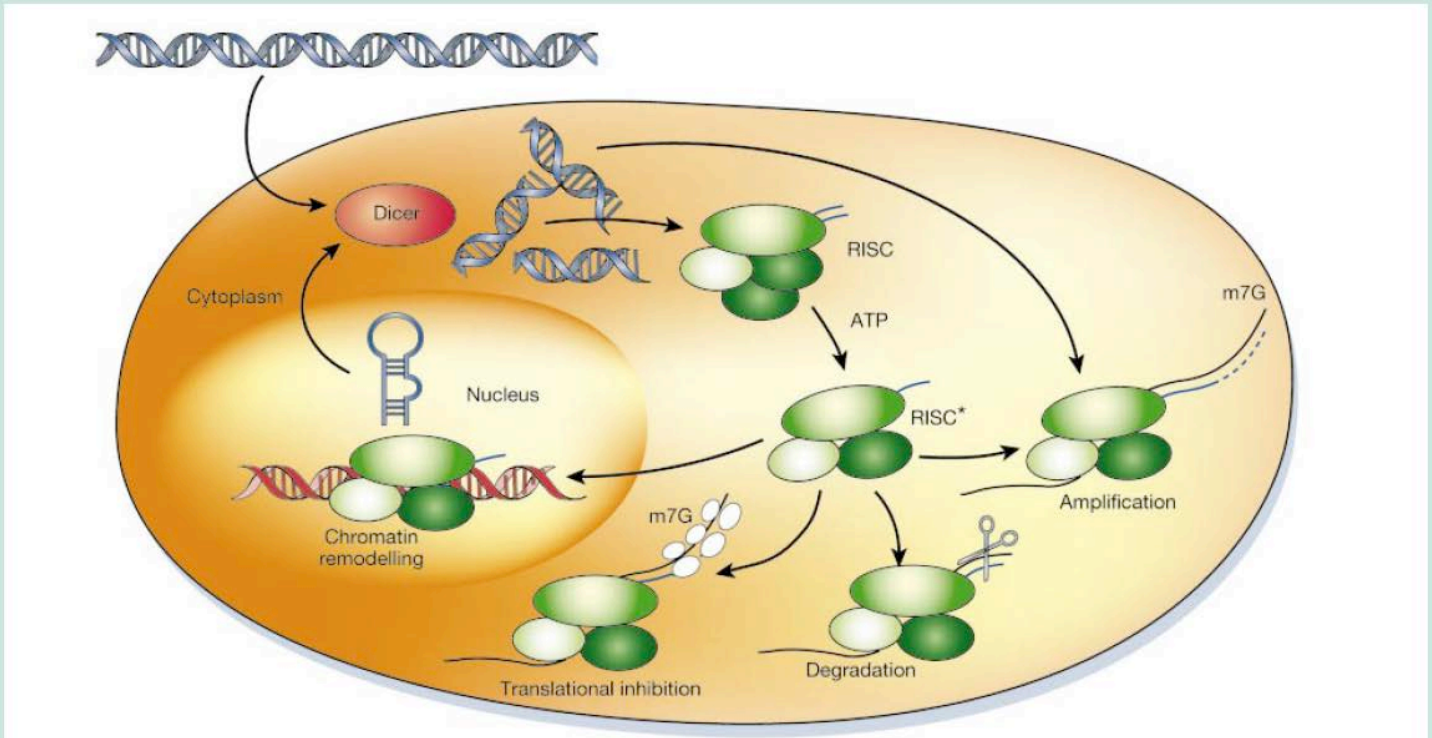
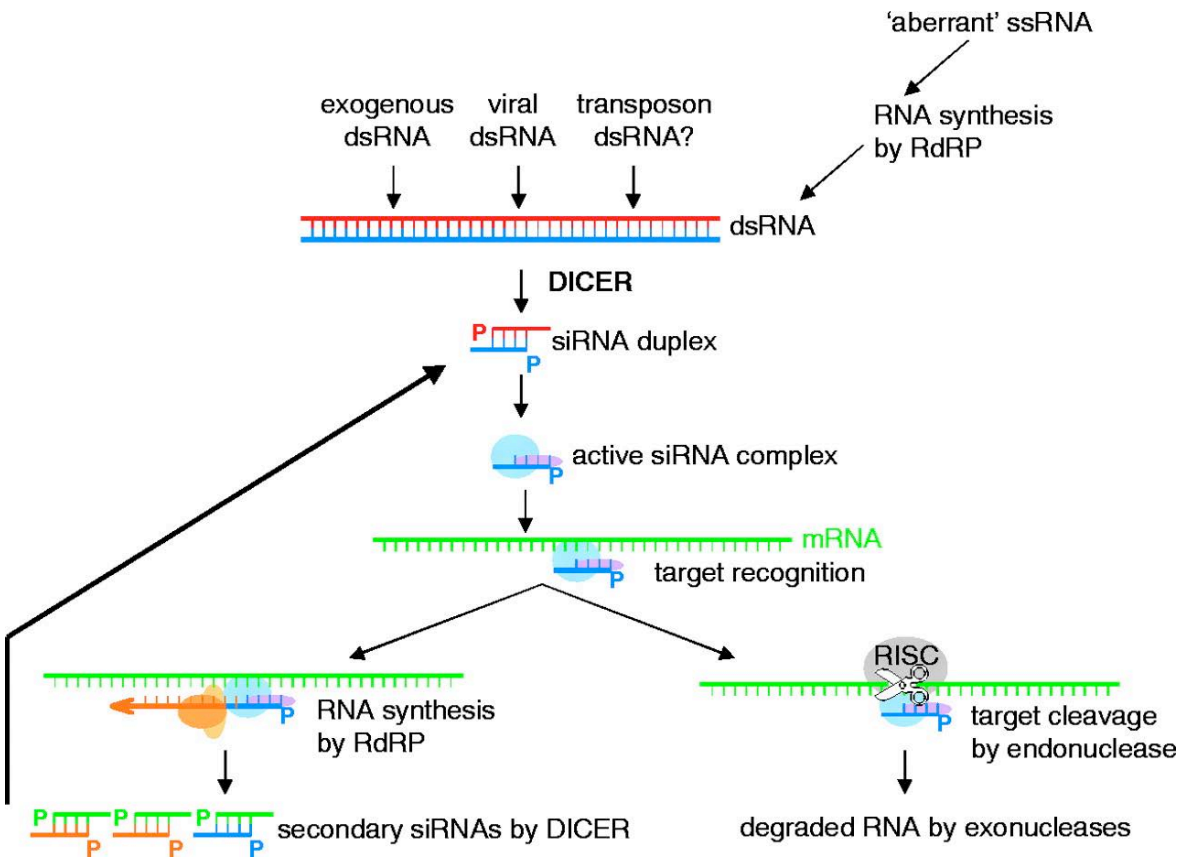


Figure 5 A model for the mechanism of RNAi. Silencing triggers in the form of double-stranded RNA may be presented in the cell as synthetic RNAs, replicating viruses or may be transcribed from nuclear genes. These are recognized and processed into small interfering RNAs by Dicer. The duplex siRNAs are passed to RISC (RNA-induced silencing complex), and the complex becomes activated by unwinding of the duplex. Activated RISC complexes can regulate gene expression at many levels. Almost certainly, such complexes act by promoting RNA degradation and translational inhibition. However, similar complexes probably also target chromatin remodelling. Amplification of the silencing signal in plants may be accomplished by siRNAs priming RNA-directed RNA polymerase (RdRP)-dependent synthesis of new dsRNA. This could be accomplished by RISC-mediated delivery of an RdRP or by incorporation of the siRNA into a distinct, RdRP-containing complex.

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://imgenex.com/rnai_anim.php

More acronyms: RdRP = RNA-dependent RNA polymerase

RNA-induced silencing complex = RISC

siNRA = small interfering RNA

What makes a ssRNA aberrant?

How does it work? Although mechanims 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

Amplifying the RNAi signal

Science 296: 1271

Figure 2. (A) Degradative and synthetic pathways linking dsRNA, siRNAs, and target mRNA in RNA silencing. Black arrows denote classical RNA silencing degradative pathways. Yellow arrows denote RdRp-dependent synthetic pathways leading to generation or amplification of dsRNA. RdRp may act on siRNA-primed dsRNA (1), siRNA-primed mRNA (2), or asRNA-primed (as=antisense) mRNA (3) to generate or amplify some or all of the inducing dsRNA sequences