

RNAi Techniques



Dr.G.MATHAN

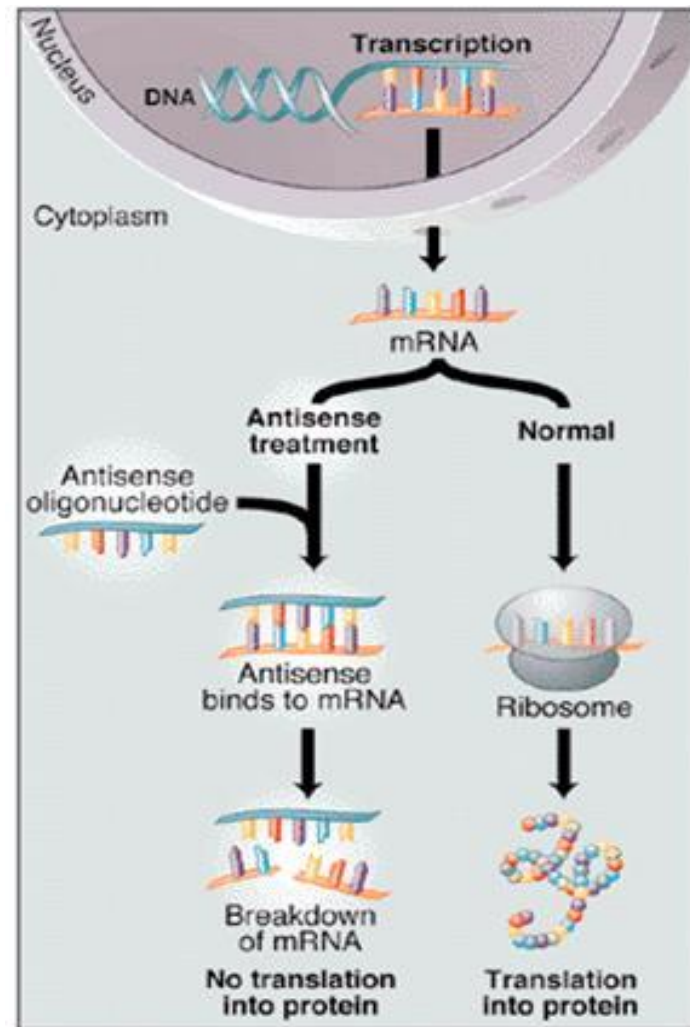
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What is the antisense oligonucleotides?

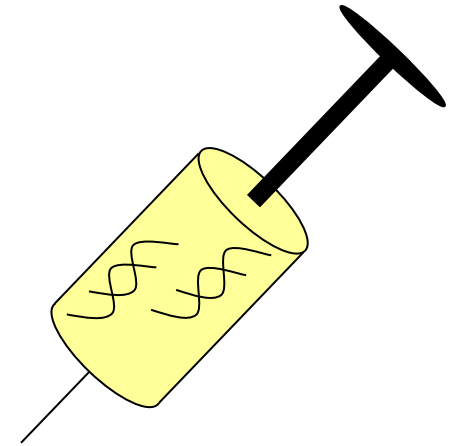


Antisense oligonucleotides ("ASOs") are synthetic polymers: The monomers are chemically-modified deoxynucleotides like those in DNA or ribonucleotides like those in RNA.

The discovery of RNA-mediated interference

- Antisense technology was used in worms...
- Difficult to explain: sense and antisense RNA preparations are each sufficient to cause interference.

inject worms with dsRNA
corresponding to a gene
involved in wiggling (*unc-22*)



QuickTime™ and a GIF decompressor are needed to see this picture.

Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

**Andrew Fire^{*}, SiQun Xu^{*}, Mary K. Montgomery^{*},
Steven A. Kostas^{*†}, Samuel E. Driver[‡] & Craig C. Mello[‡]**

The discovery of RNA-mediated interference

Working hypothesis: the silencing effect by antisense or sense RNA might be due to low-level contaminations of **double-stranded RNA**.

Gene	segment	Size (kilobases)	Injected RNA	F ₁ phenotype
				<i>unc-22</i> -null mutants: strong twitchers ^{7a}
<i>unc22A</i>	Exon 21-22	742	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
<i>unc22B</i>	Exon 27	1,033	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
<i>unc22C</i>	Exon 21-22†	785	Sense + antisense	Strong twitchers (100%)

Results:

- double-stranded RNA is far more effective than single-stranded RNA.
- The sense or antisense RNAs lose their silencing effect if they are purified from the contaminating Double-stranded RNA (dsRNA).
- only a few molecules of dsRNA are required per cell → non-stoichiometric effect that implies an amplification component.

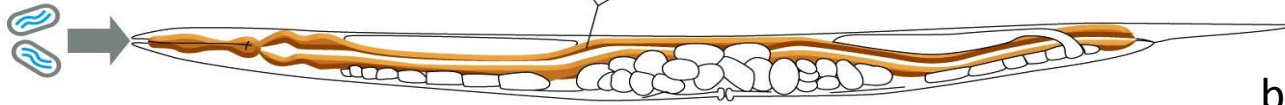
RNAi –Rapid way to test Gene Function

RNAi used to inactivate genes in Drosophilla and mammalian cell culture lines

To study the consequences and importance of the particular gene function through this study

Dominant negative mutation created by RNAi

1 *E. coli*, expressing double-stranded RNA, eaten by worm



2 double-stranded RNA injected into gut

A. Double stranded RNA introduced in to *C. elegans*

1. Either by feeding the *E. coli* expressing dsRNA or
2. By injecting directly in to gut

It inhibits the expression of target gene in different tissue TYPE

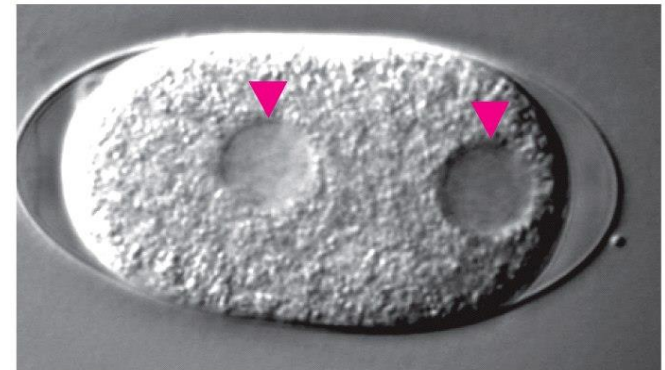
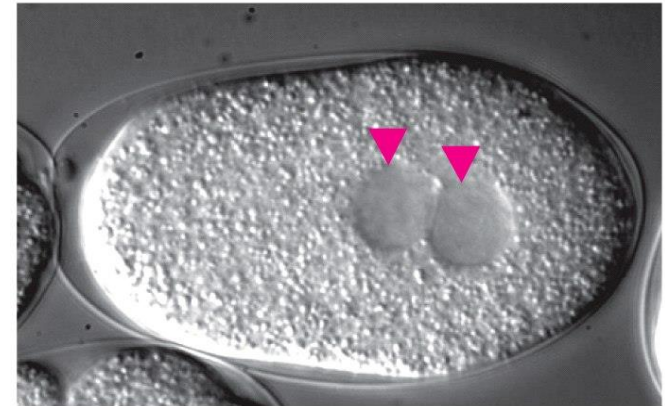
- The RNAi effect is capable of **spreading** within the whole nematode.

→ this observation led to new discoveries that allowed the identification of genes and Therefore to the mechanisms that are involved in RNAi

B. Wild type worm-embryo after the egg has been fertilized
Egg and sperm pronuclei (red arrow) migrated and come Together in the posterior half of the embryo

c. Worm embryo at the same stage in which a gene involved In cell division has been inactivated by RNAi
2 pronuclei have failed to migrate

b

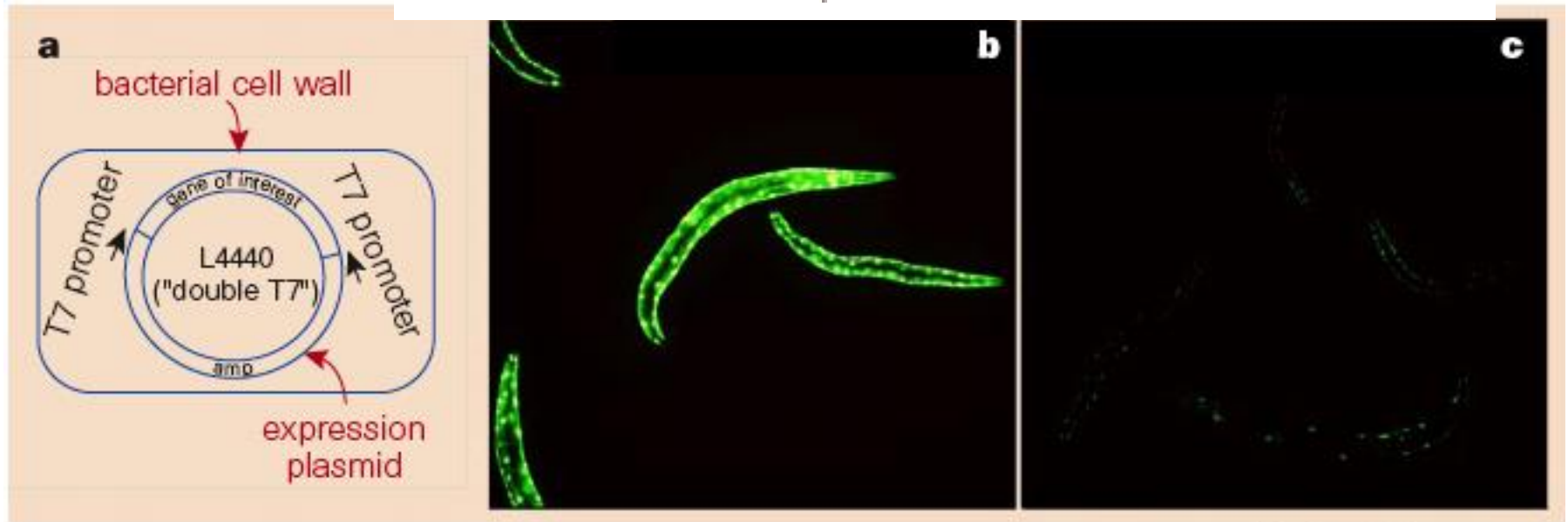
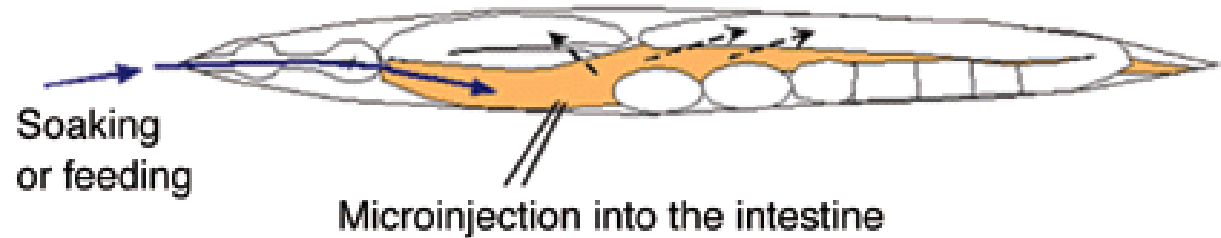


c

20 μ m

RNAi does not require microinjection

Transgenes and endogenous genes can be inactivated through soaking worms in dsRNA or by feeding them with bacteria that produce dsRNA.

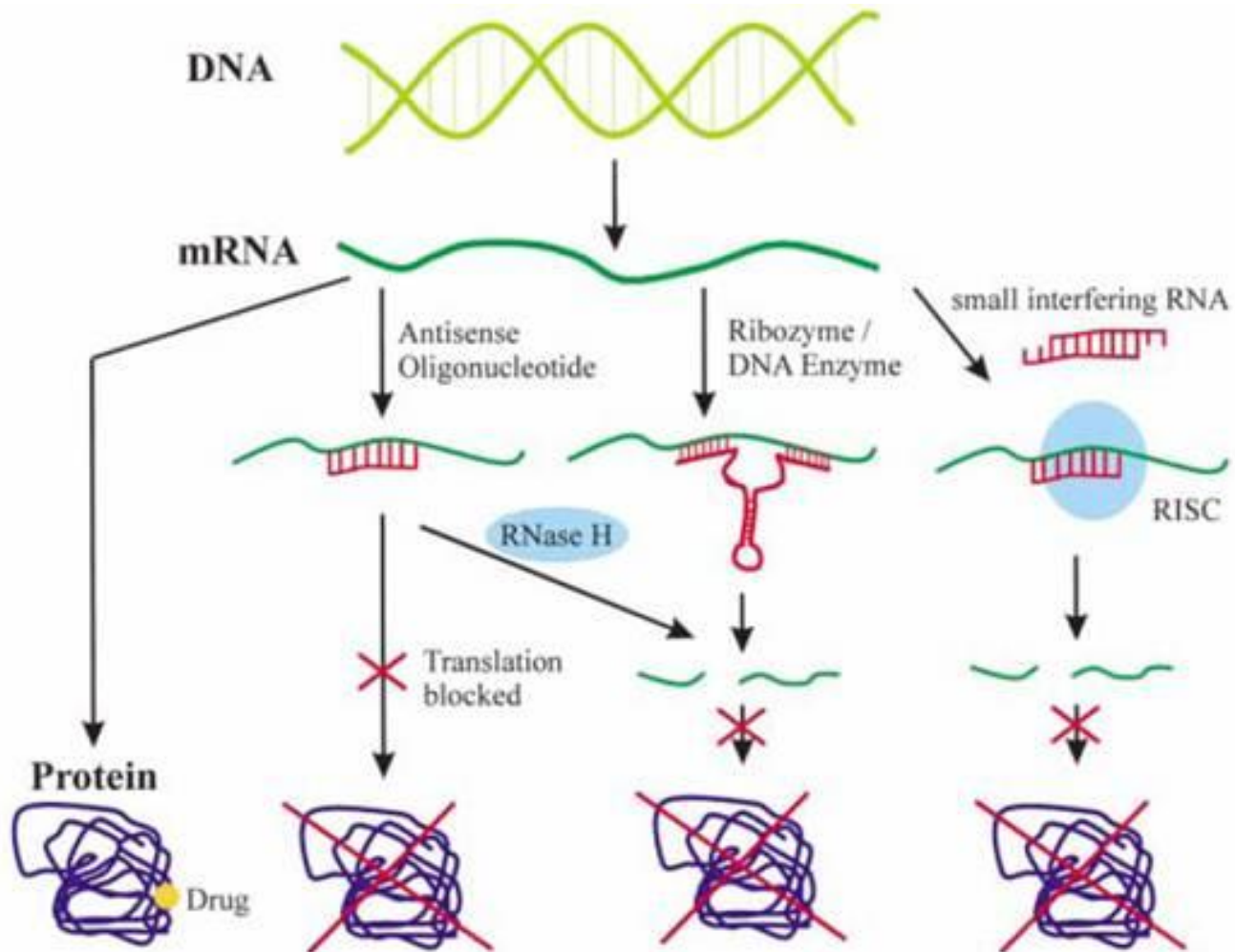


GFP-expressing strain fed on naive bacteria (b) and on dsRNA production in bacteria bacteria expressing dsRNA corresponding to the gfp coding region

Timmons and Fire, Nature **395**. (1998)

Tabara et al, Science **282** (1998)

Anti-mRNA Strategies

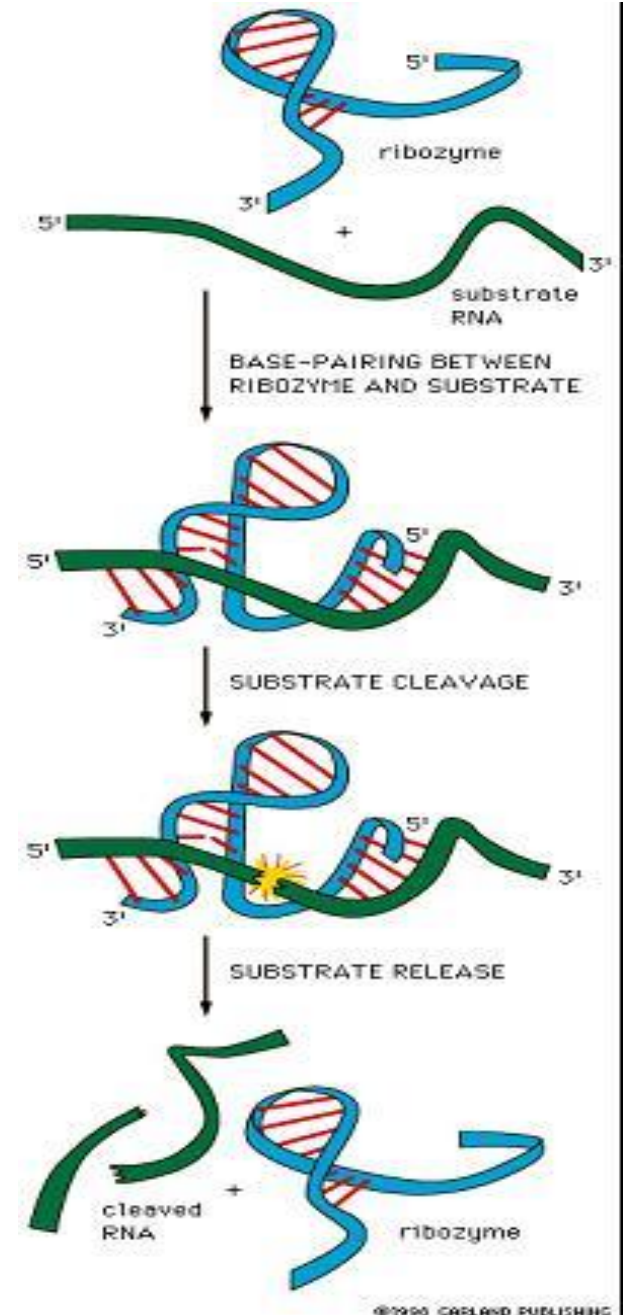


Ribozymes

- Ribozymes are RNA molecules that catalyze biochemical reactions.

1982 T. Cech shows that the intron of one Tetrahymena pre-rRNA is self splicing. He proposes the term “ribozyme” to refer to catalytic RNA

- Ribozymes cleave single-stranded regions in RNA through transesterification or hydrolysis reactions that result in cleavage of phosphodiester bonds



Limitations of Practical Applications of Antisense Oligonucleotides

Despite the simplicity of the idea behind the Antisense, several problems have to be overcome for successful application:

1. Accessible sites of the target RNA for oligonucleotide binding have to be identified.
2. Antisense agents have to be protected against nucleolytic attack.
3. Cellular uptake and correct intracellular localization.

RNAi: Double-Stranded RNA Directs the ATP-Dependent Cleavage of mRNA at 21 to 23 Nucleotide Intervals

Phillip D. Zamore,^{*#} Thomas Tuschl,^{†#}
Phillip A. Sharp,^{‡§} and David P. Bartel^{¶||}

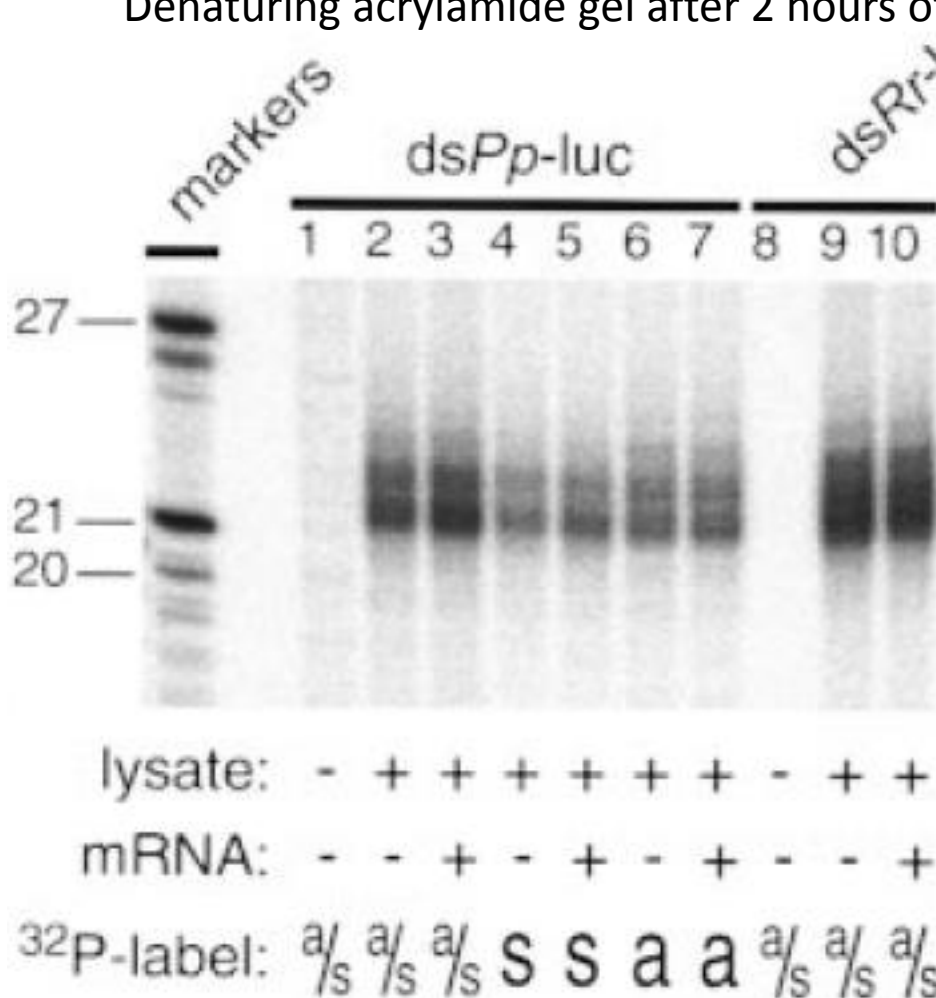
During the RNAi reaction, both strands of the dsRNA are processed to RNA segments 21-23 nucleotides in length.

Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA.

The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21-23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21-23 nucleotide fragments from the dsRNA are guiding mRNA cleavage

21-22 nt RNA fragments are produced

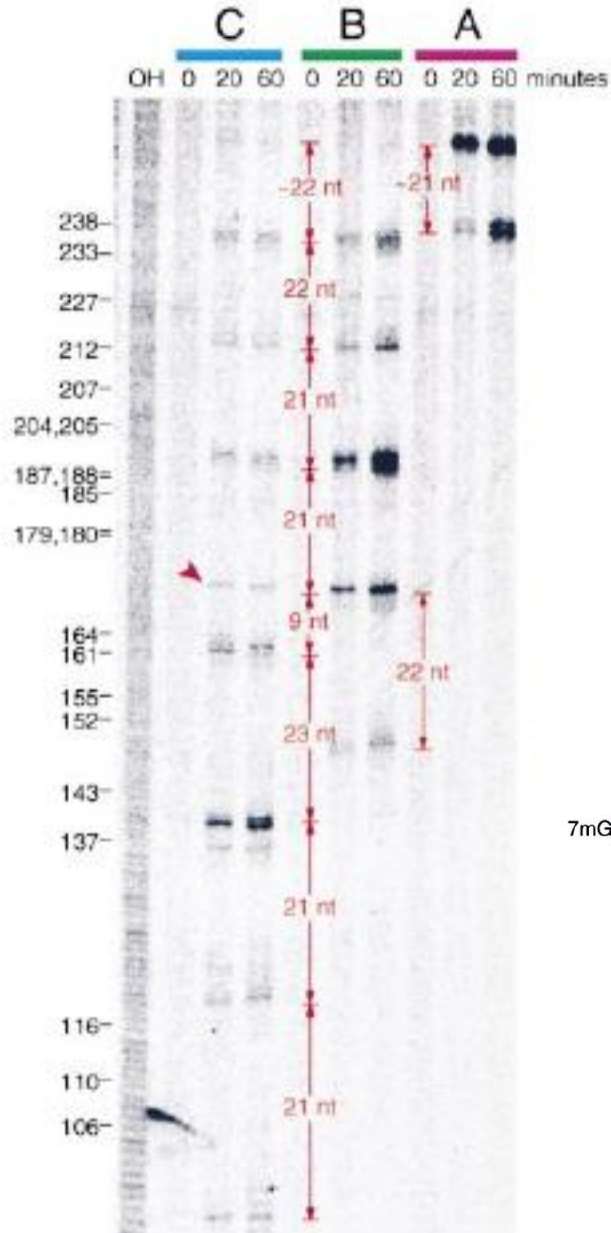
Experiment: *Drosophila* cell lysate + labelled dsRNA (one or both strands).
Denaturing acrylamide gel after 2 hours of incubation.



Results:

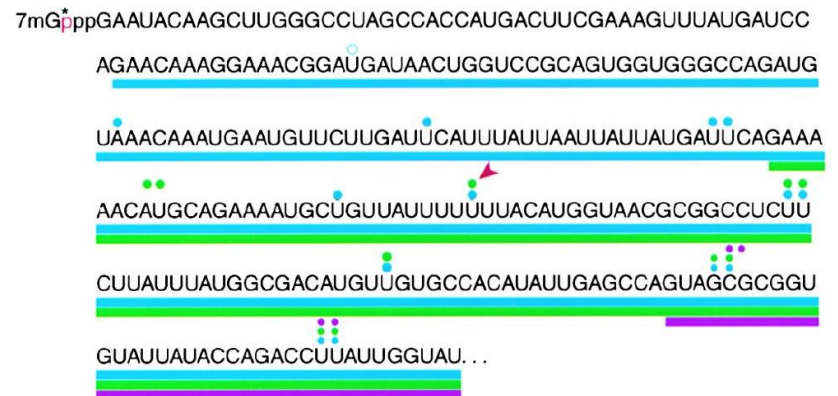
- 21-mer formation does not require the presence of the corresponding mRNA.
- both strands are equally processed.

The target mRNA is cleaved in 21-23 nt intervals

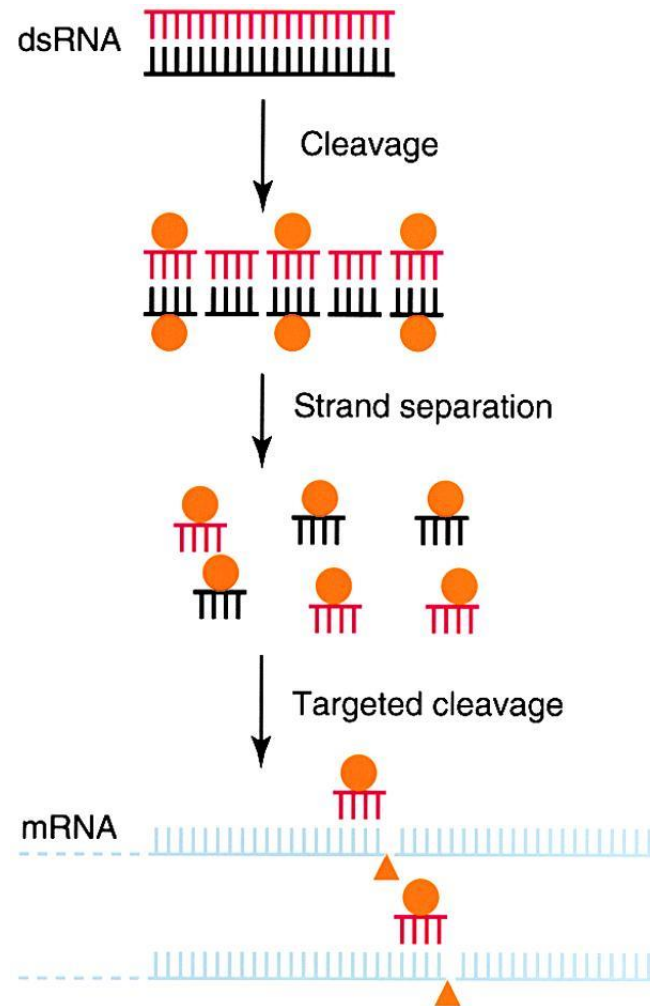


3 different target RNAs were tested (A, B, C).

Intermediate digestion products show differences of 21-23 nt.



A first model for the mechanism RNAi



Zamore et al, Cell, v101 pp25-33 (2000)

miRNA

MicroRNAs (miRNAs) are genomically encoded non-coding RNAs that help regulate gene expression, particularly during development.

Mature miRNAs are structurally similar to siRNAs produced from exogenous dsRNA, but before reaching maturity, miRNAs must first undergo extensive post-transcriptional modification.

An miRNA is expressed from a much longer RNA-coding gene as a primary transcript known as a *pri-miRNA* which is processed, in the cell nucleus, to a 70-nucleotide stem-loop structure called a *pre-miRNA* by the microprocessor complex.

MicroRNAs (miRNAs) are endogenous approximately 22 nt RNAs that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression.

miRNA processing and mechanism

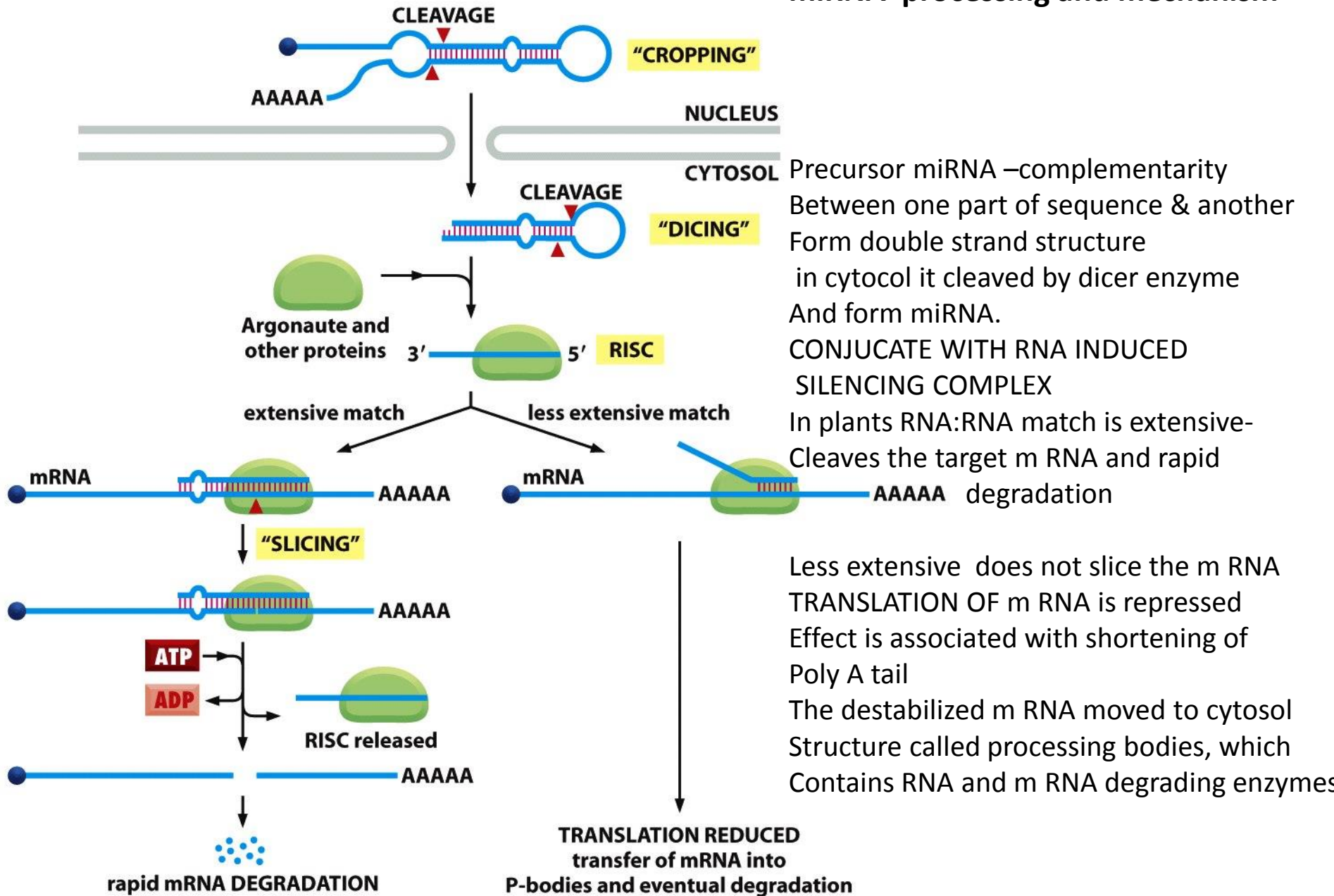
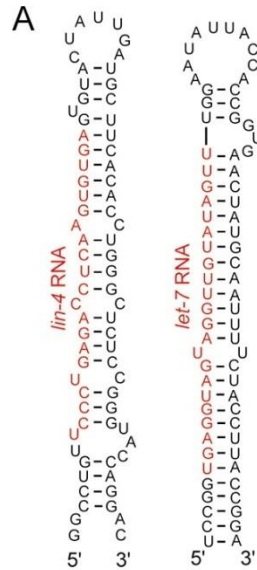
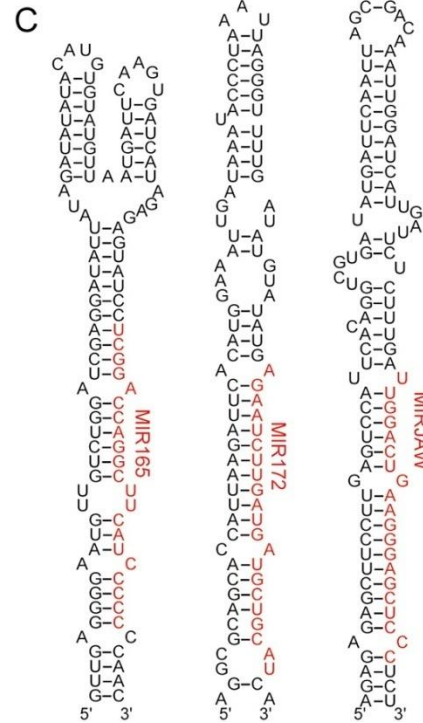
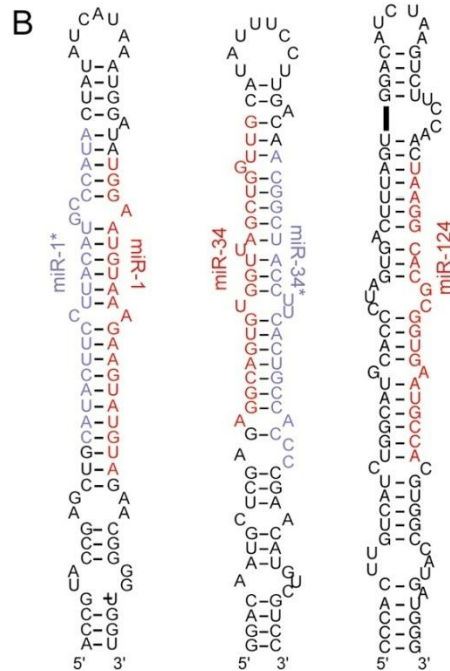


Figure 7-112 *Molecular Biology of the Cell* (© Garland Science 2008)

First miRNA in *C.elegans*



miRNA in *C.elegans* with homologs In flies and human



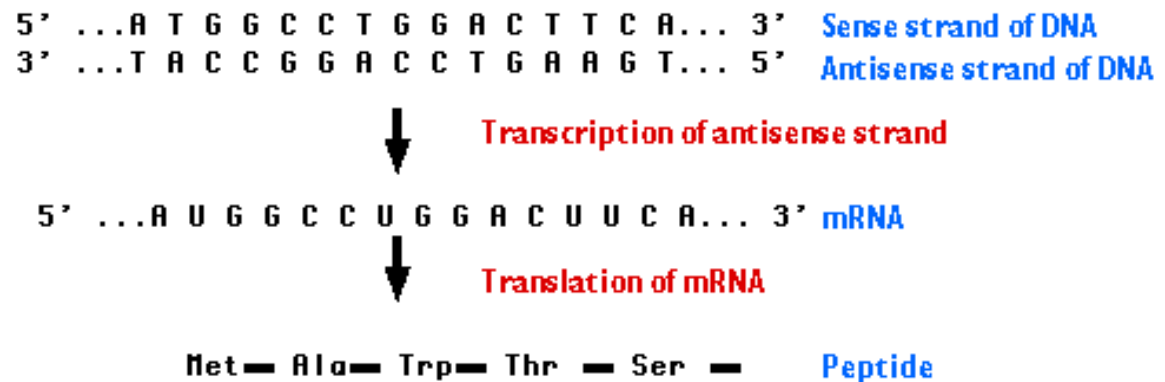
Human: 200-255 miRNA

C.elegans: 103-120 miRNA

Drosophila: 96-124 miRNA

miRNA in Plants

What are sense and antisense RNA?



5' C U U C A 3' mRNA
3' G A A G U 5' Antisense RNA

- Messenger RNA (mRNA) is single-stranded, called "sense"

RNAi terms

- **dsRNA: double stranded RNA, longer than 30 nt**
- **miRNA: microRNA, 21-25 nt.**
 - **Encoded by endogenous genes**
- **siRNA: small-interfering RNA, 21-25 nt.**
 - **Mostly exogenous origin**

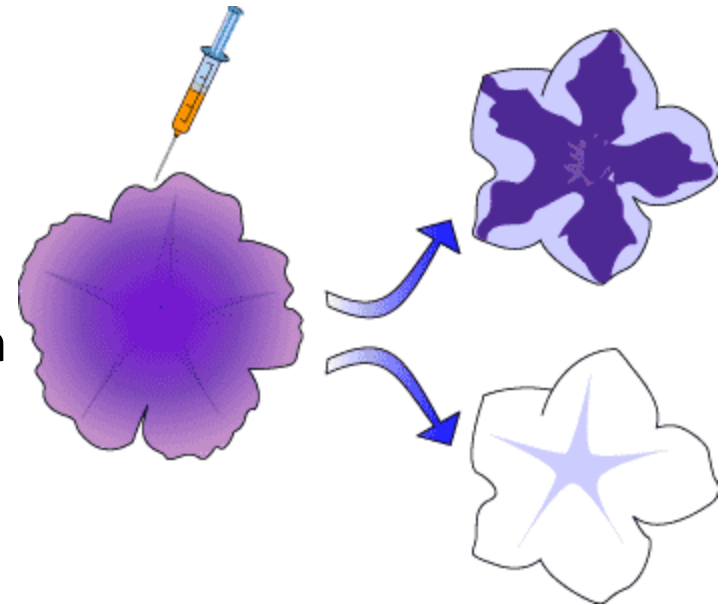
Alternate terms to RNAi

- **PTGS (Posttranscriptional Gene Silencing)**
- **Cosuppression**
- **Quelling**
- **Virus-induced gene silencing**

Overexpression of chalcone synthase in petunias unexpectedly resulted in white petunias



- Napoli et al. defined an RNAi-like phenomenon and called it *“cosuppression”*.
- chalcone synthase (CHS), a key enzyme in flavonoid biosynthesis, the rate-limiting enzyme in anthocyanin biosynthesis, responsible for the purple coloration
- The levels of endogenous as well as introduced CHS were 50-fold lower than in wild-type petunias, which led the authors to hypothesize that the introduced transgene was **“cosuppressing”** the endogenous CHS gene.



The Laureates

Andrew Fire, born in 1959, is a US citizen. Since 2003 he has been professor of Pathology and Genetics at Stanford University School of Medicine, Stanford, California, USA.

In 1983 he took his PhD in Biology at the Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. He began his research on the nematode *C. elegans* during his time as visiting scientist in Cambridge, England, at the laboratory of Sydney Brenner (Nobel Laureate 2002). When Fire and Mello made their key discoveries about RNA interference, Fire was working at the Carnegie Institution of Washington.



L. CICERO/STANFORD



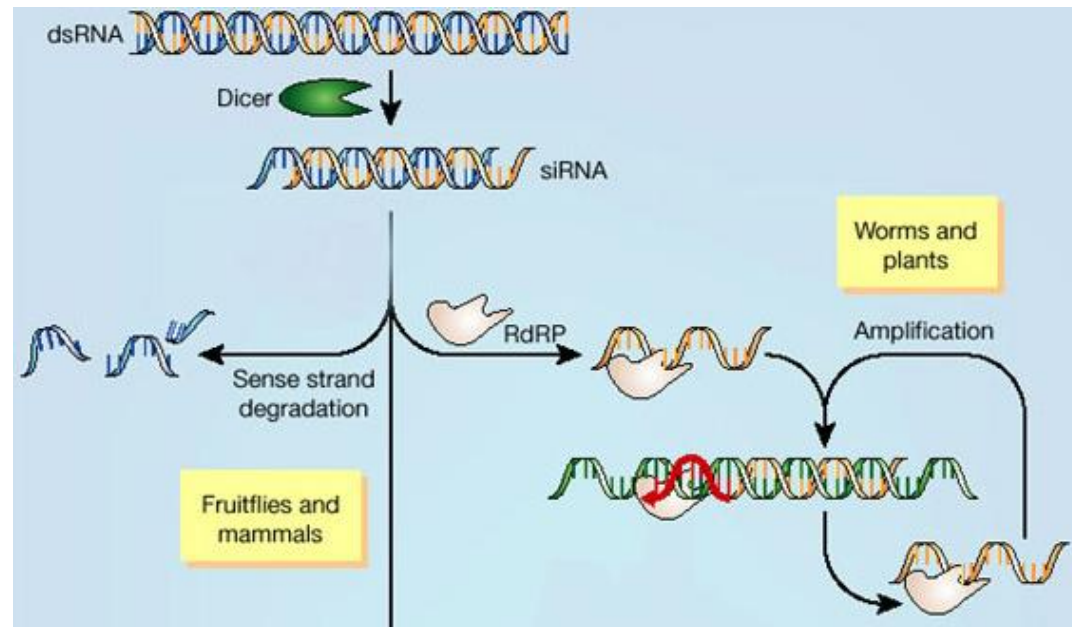
R. CARLINI/MINAS

Craig Mello, born in 1960, is a US citizen and a professor of Molecular Medicine. Since 1994 he has worked within the Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts, USA. He is also a Howard Hughes Medical Institute Investigator.

In 1990 he took his PhD in Cellular and Developmental Biology at Harvard University, Boston, Massachusetts. Before he moved to the University of Massachusetts Medical School in Worcester, he worked at the Fred Hutchinson Cancer Research Center.

siRNA biogenesis

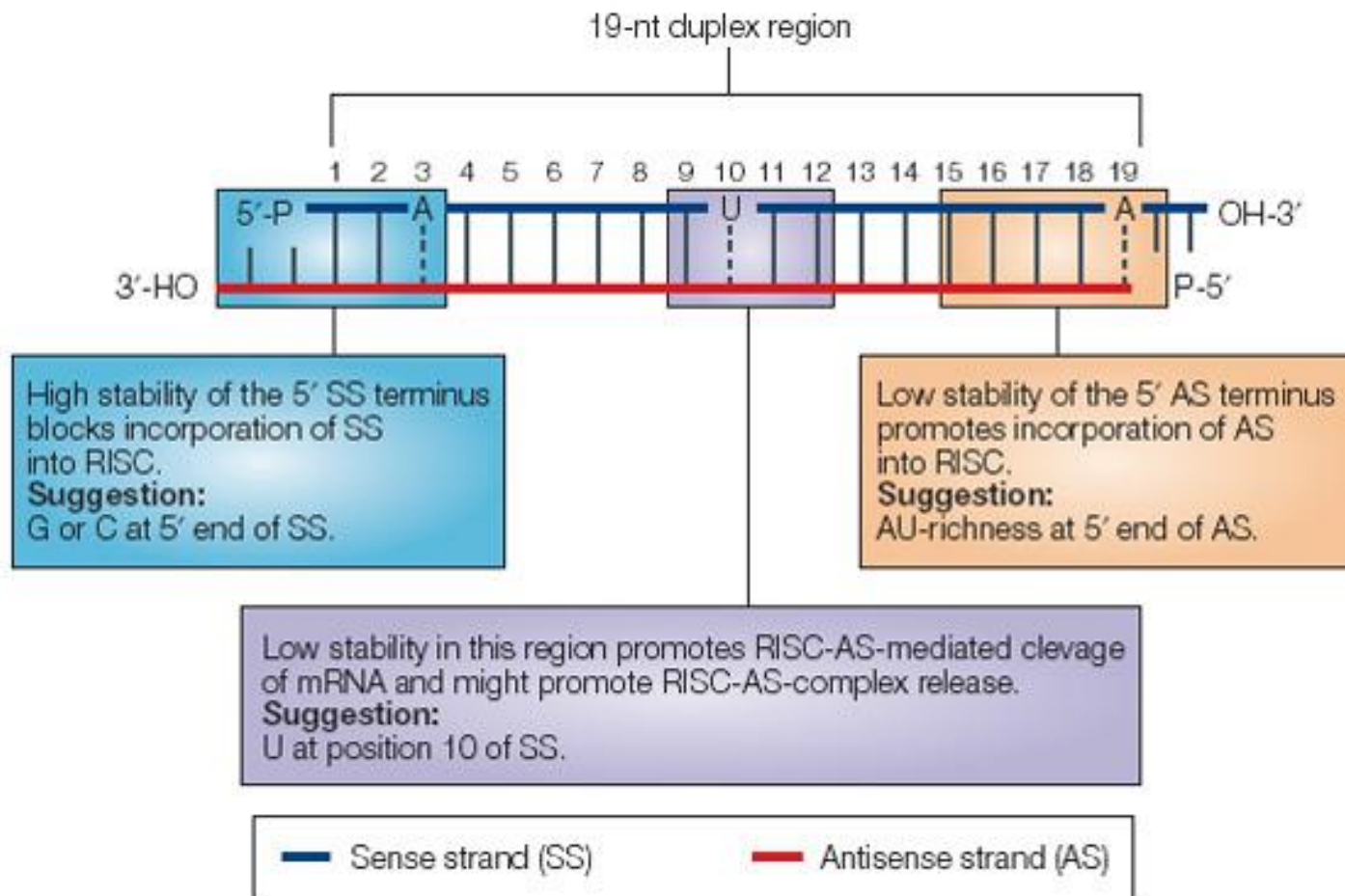
- Dicer (type III RNase III) cleaves long dsRNA into siRNA 21-25nt dsRNA from exogenous sources
 - Symmetric 2nt 3' overhangs, 5' phosphate groups
 - Evidence for amplification in *C. elegans* and plants



RNA Induced Silencing Complex (RISC)

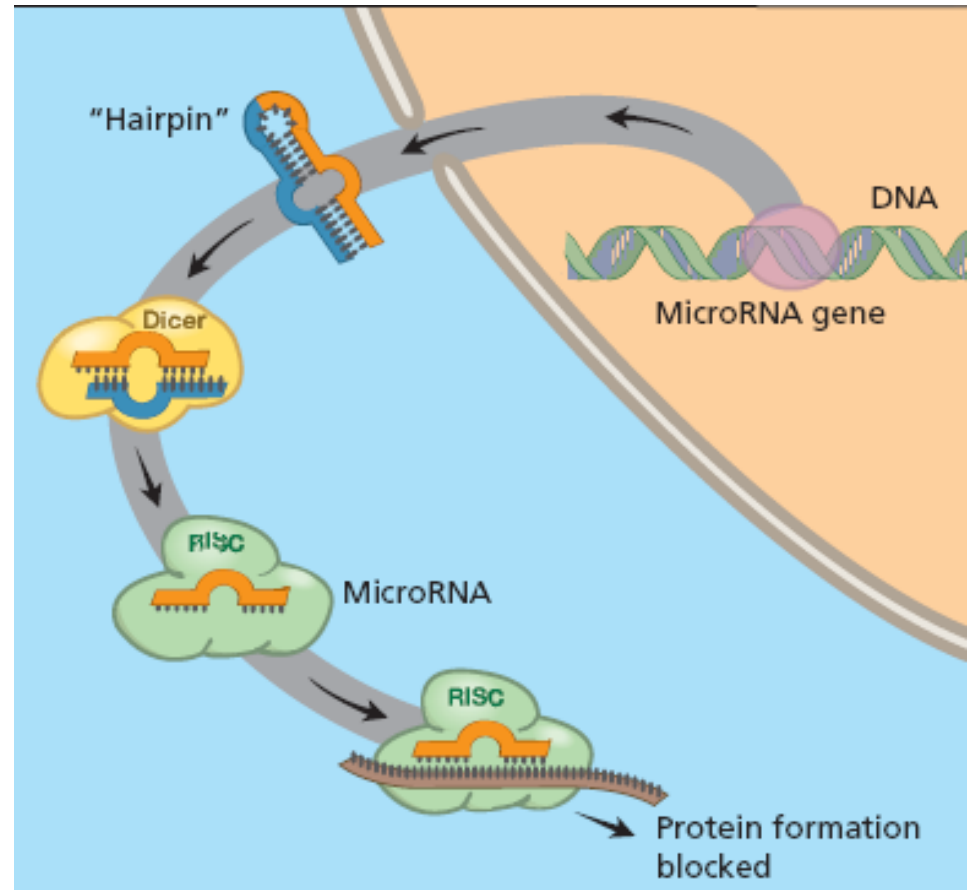
- RNAi effector complex
- Preferentially incorporates one strand of unwound RNA [Khvorova et al., 2003]
 - Antisense
- How does it know which is which?
 - The strand with less 5' stability usually incorporated into RISC [Schwarz et al., 2003]

siRNA design

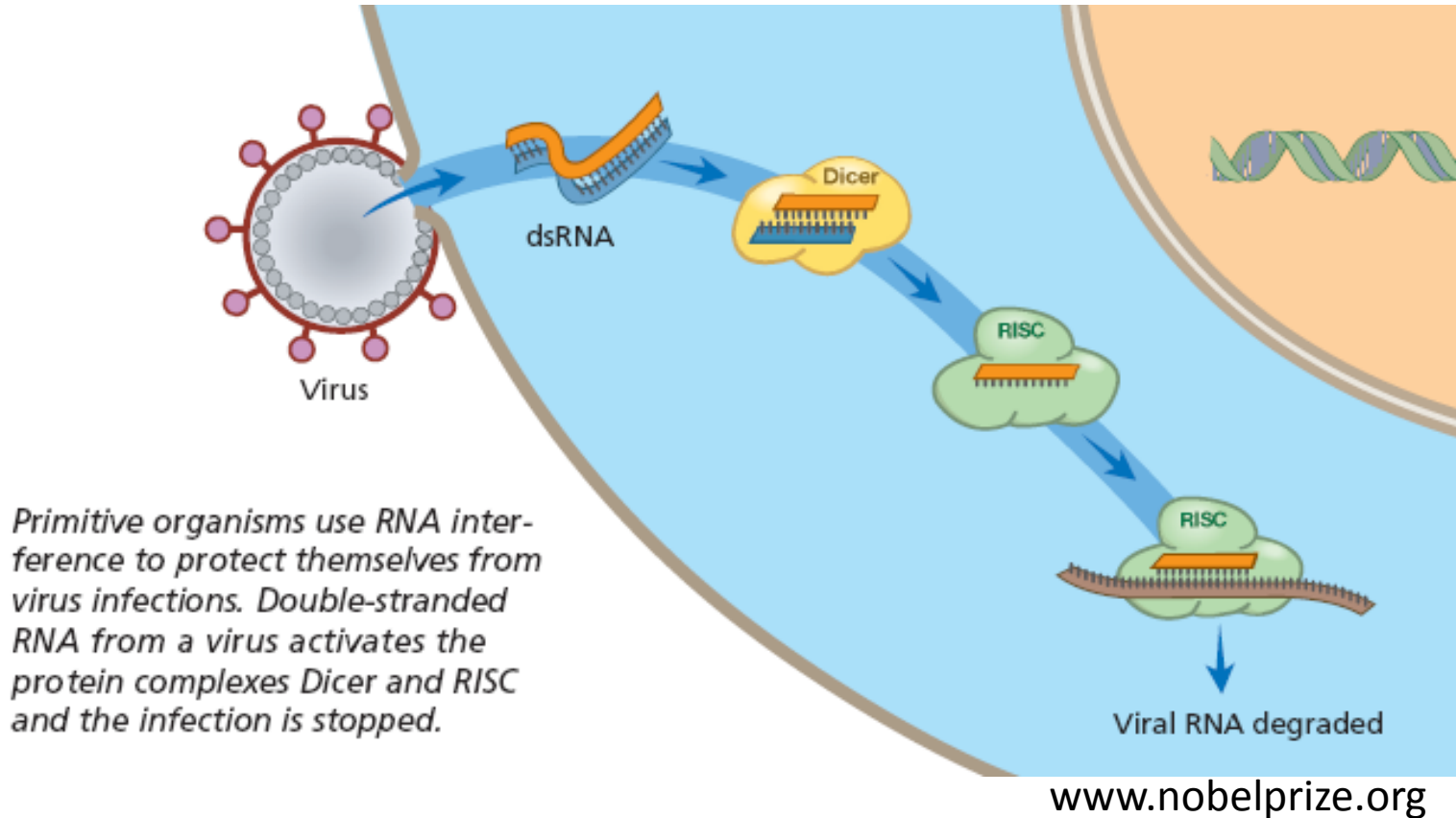


Endogenous RNAi-miRNA

- We have hundreds of different genes that encode small RNA (collectively, microRNA) whose precursors can form double-stranded RNA. These can activate the RNA interference process and thus switch off the activity of various genes with matching segments.
- First miRNA is lin-4

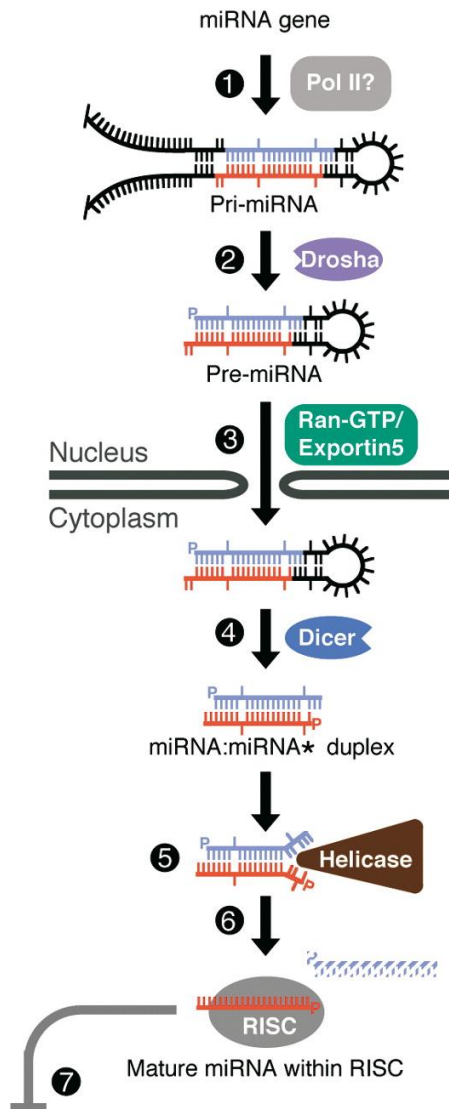


Defense Against Viruses

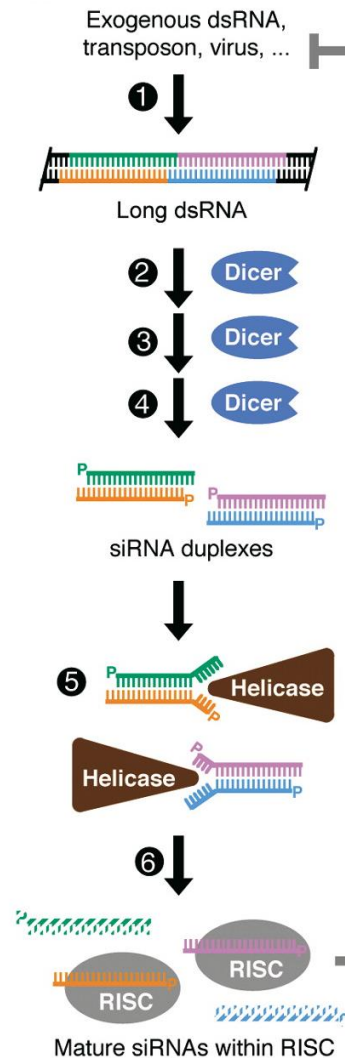


■ Indeed, Baulcombe, Vance, and others have shown that, in the continuing evolutionary war to survive and reproduce, plant viruses have evolved genes that enable them to suppress silencing.

miRNA



siRNA



A. miRNAs are produced by the successive actions of two RNaseIII ribonucleases:

Drosha (RNase) and Pasha (dsRNA binding protein) act as a heterodimeric complex in the nucleus.

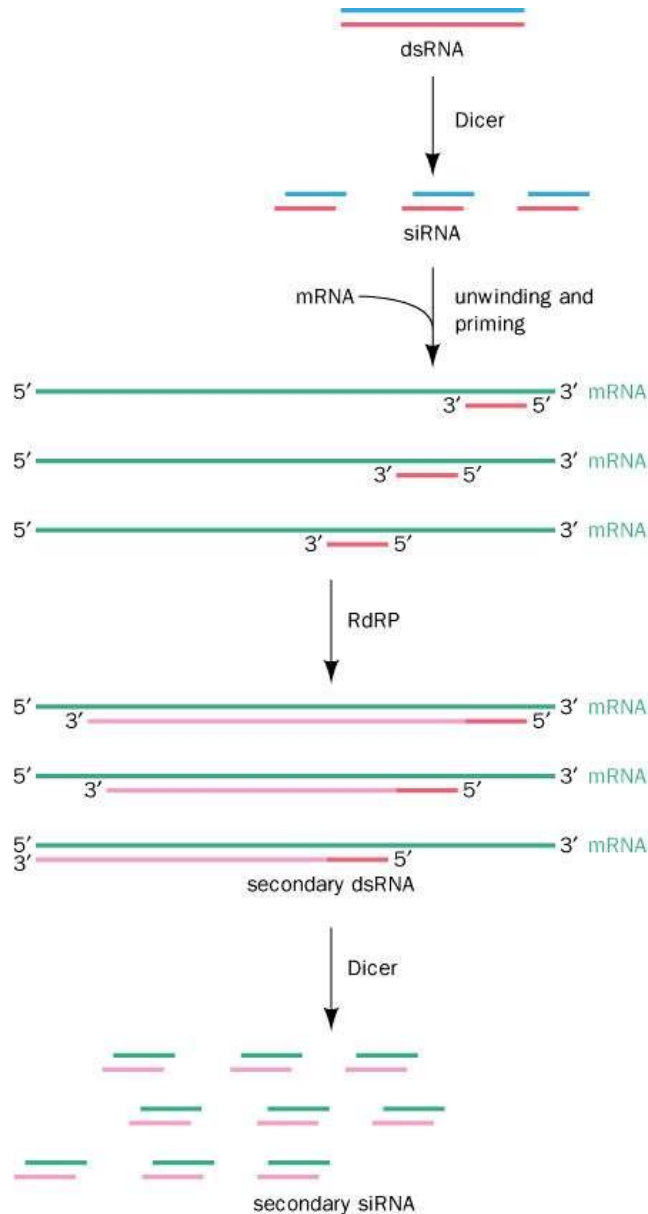
The resulting pre-miRNA is then exported into the cytoplasm where it is cleaved by Dicer.

The miRNA duplex must be unwound and the miRNA strand be incorporated into RISC.

B. long dsRNA is a substrate for Dicer but not for Drosha. Dicer cut two times to yield the siRNA duplex.

The guide strand is incorporated into RISC.

A model for transitive RNAi



1) siRNA resulting from the action of Dicer is unwound and binds to its target mRNA.

2) There it acts as a primer for RNA-dependent RNA polymerase (RdRP).

3) 5'-3' extension.

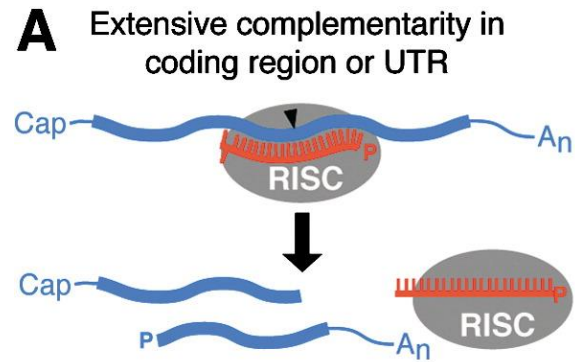
4) The resulting secondary dsRNA is then cleaved by Dicer to form secondary siRNAs.

→ amplification of the siRNA

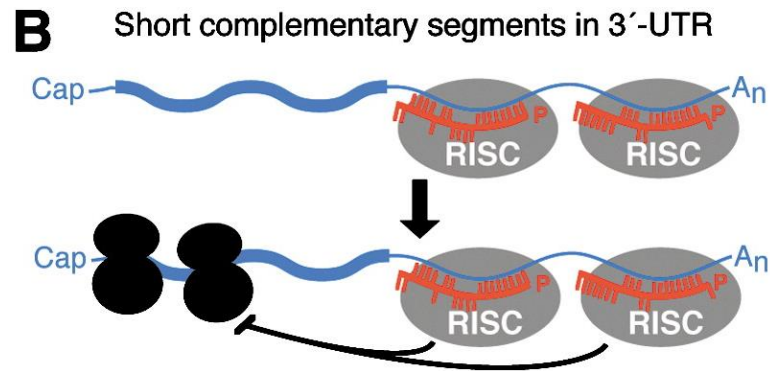
→ genes that are located 5' to the initial target gene may also be silenced.

The effect is limited to 300-500 nt 5' to the initial target site.

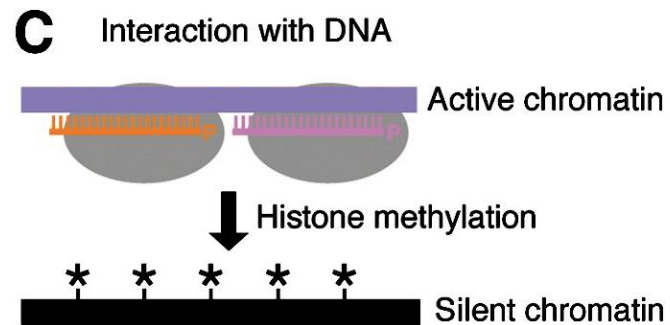
Post-transcriptional
Cleavage of mRNA



Translational repression
of the mRNA



Transcriptional
silencing



Designing siRNA

- The target sequence should be 50-100 bp downstream of start codon or in the 3' UTR
- Search for a 23nt long sequence
- Ensure that your target sequence is not homologous to any other genes
- Avoid more than three guanosines or three cytosines in a row
- avoid stretches of > 4 T's or A's
- secondary structure of the target mRNA does not appear to have a strong effect on silencing
- Designing several siRNA's helps to find a highly efficient one

Example for siRNA's

CEP

targeted region (cDNA): 5' AACTGGACTTCCAGAAGAACATC

sense siRNA: 5' CUGGACUUCCAGAAGAACAdt

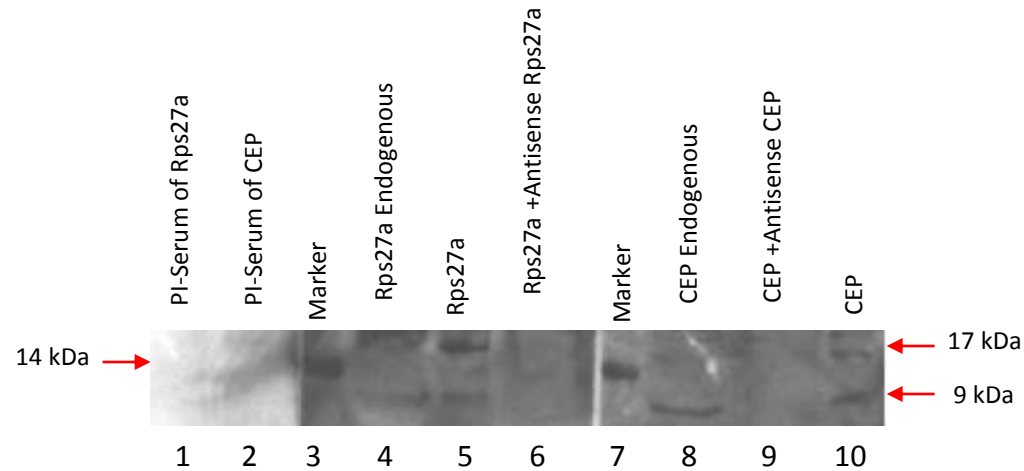
antisense siRNA: 5' UGUUCUUCUGGAAGUCCAGdt

RPS27a

targeted region (cDNA): 5' AACGTACGCGGAATACTTCGATT

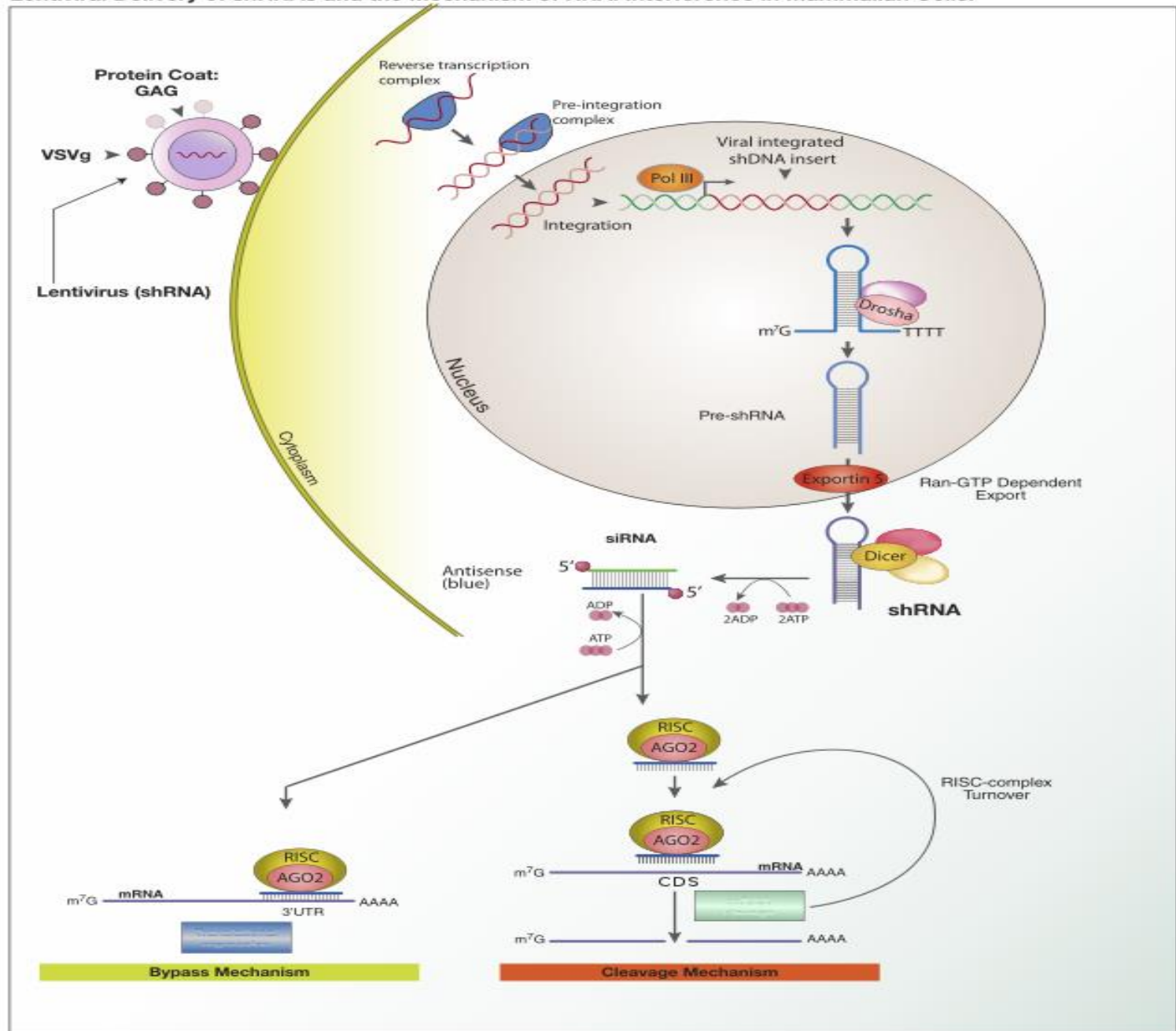
sense siRNA: 5' CGUACGCGGAAUACUUCGAdTdT

antisense siRNA: 5' UCGAAGUAUUCCGCGUACGdTdT



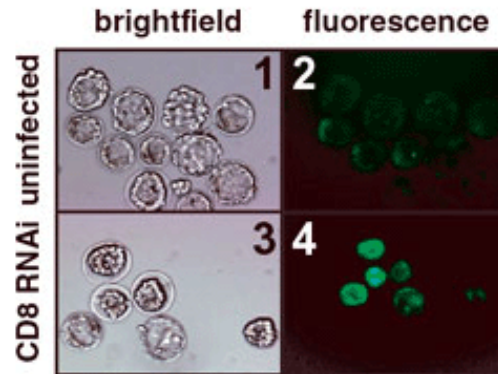
Detection of Rps27a and CEP expression in Huh7 cell lines. Total cells were lysed after 48hr posttransfection with Rps27a (lanes 5), anti-Rps27a (lanes 6), CEP (lanes 10) and anti-CEP (lanes 9).

Lentiviral Delivery of shRNAs and the Mechanism of RNAi Interference in Mammalian Cells.



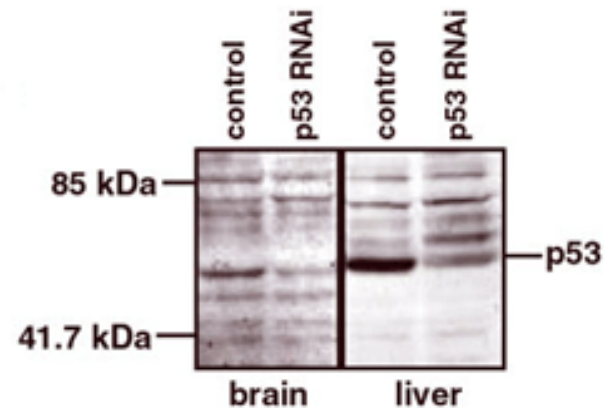
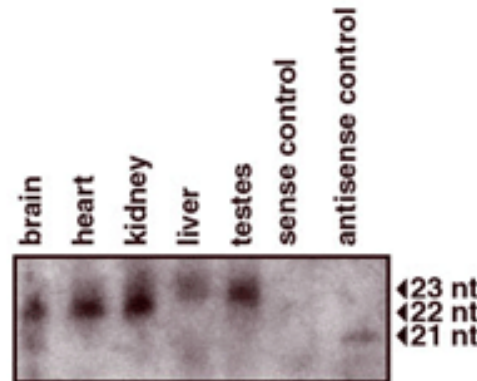
Functional silencing of genes in mice by Lentivirus-infection

Generation of lentivirus
infected zygotes



Silencing of p53:

Tissue was harvested
from 8-wk-old mice



RNA interference in biotechnology

Engineering food plants.

For example, cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid product gossypol, making them unsuitable for human consumption.

RNAi has been used to produce cotton stocks whose seeds contain reduced levels of delta-cadinene synthase, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where gossypol is important in preventing damage from plant pests.

Similar efforts have been directed toward the reduction of the cyanogenic natural product linamarin in cassava plants.

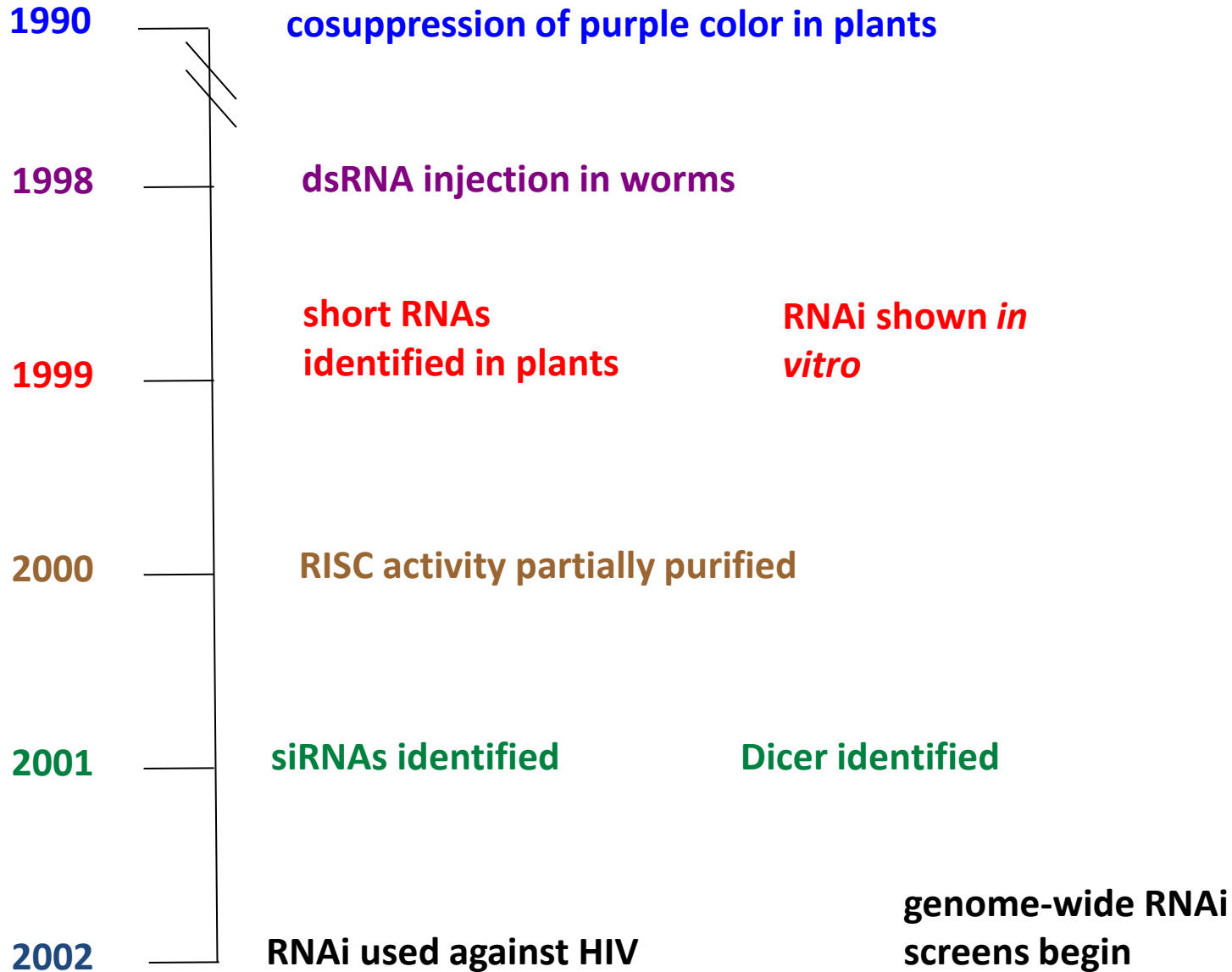
Clinical Trials of Antisense Oligonucleotides

Table 4: Antisense Compounds Approved or in Clinical Trials (completion based on references 7,12,33,205)

Product	Company	Sequence	Chemistry	Target	Disease	Route of Administration	Status (Phase)
Vitravene™	Isis/Novartis	GCGTTTGCTCTTCTTCTTGCG	PS	IE2	CMV Retinitis	Intravitreal	On Market
Affintak™	Isis/Lilly	GTTCTCGCTGGTGAGTTTCA	PS	PKC-α	Cancer-NSCLC, others	Parenteral	III
Alicaforsen™	Isis	GCCCAAGCTGGCATCCGTCA	PS	ICAM-1	Crohn's Disease	Parenteral	III
ISIS 2302	Isis	GCCCAAGCTGGCATCCGTCA	PS	ICAM-1	Topical Psoriasis	Topical	II
ISIS 2302	Isis	GCCCAAGCTGGCATCCGTCA	PS	ICAM-1	Ulcerative Colitis	Enema	II
ISIS 2503	Isis	TCCGTCATCGCTCCTCAGGG	PS	H-ras	Cancer-pancreatic, others	Parenteral	II
ISIS 14803	Isis/Elan	GTGCTCATGGTGACGGTCT	PS-DNA	Antiviral	Hepatitis C	Parenteral	II
ISIS 104838	Isis/Elan	GCTGATTAGAGAGAGGTCCC	2 nd Gen. Chimeric PS	TNF-α	Rheumatoid Arthritis	Parenteral/Oral	II
ISIS 104838	Isis	GCTGATTAGAGAGAGGTCCC	2 nd Gen. Chimeric PS	TNF-α	Psoriasis	Topical	II
OGX-011	Isis	N/A	N/A	Clusterin	Cancer	Parenteral	I
Genasense™	Genta	TCTCCCAGCGTGCGCCAT	PS	Bcl-2	Cancer	Intravenous	II/III
E2F Decoy	Corgentech	N/A	N/A	E2F	Atherosclerosis	Ex-vivo	II/III

antisense oligonucleotide (**fomivirsen**) to treat Cytomegalovirus (CMV) retinitis

Timeline



Acknowledgement

- ❖ The Presentation is being used for educational and non commercial purpose
- ❖ Thanks are due to all those original contributors and entities whose pictures used for making this presentation.