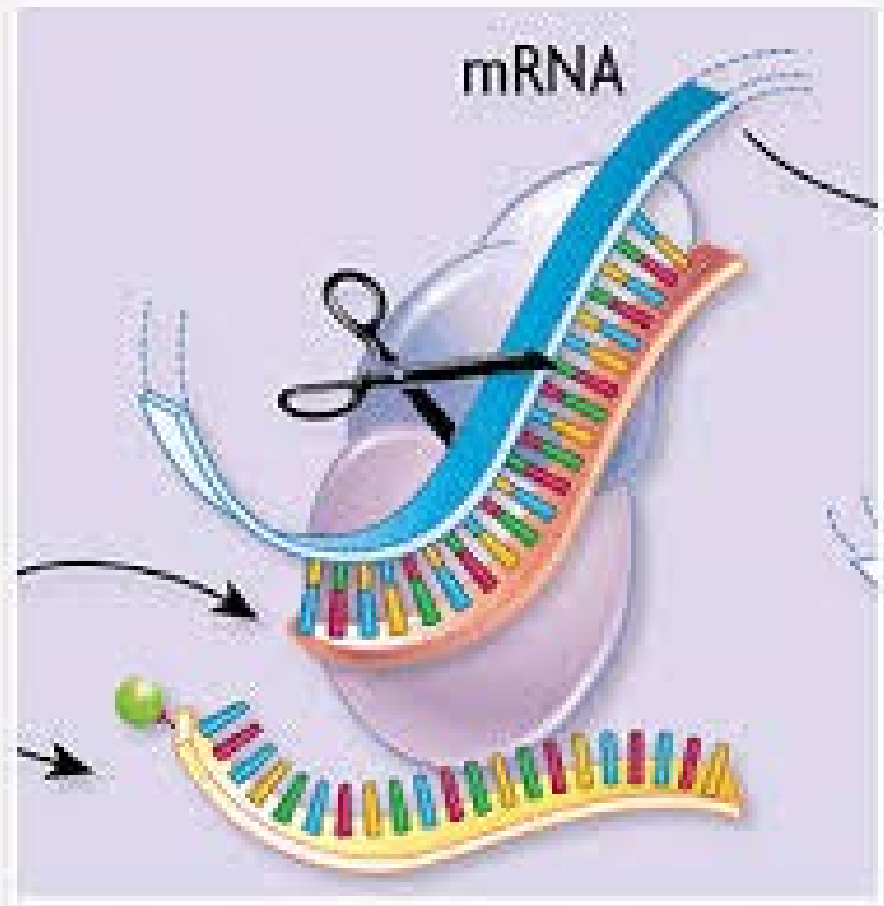
Vectors for RNAi



Phenomena first observed in petunia

Attempted to overexpress chalone synthase (anthrocyanin pigment gene) in petunia. (trying to darken flower color)

Caused the loss of pigment.

Jorgensen 1990
van der Krol 1990

Gene injection (pigmentation Enzyme-petunias)
Expectation: more red color
Co-suppression of transgene and endogenous gene.

Bill Douherty and Lindbo 1993

Gene injection with a complete tobacco etch virus particle.
Expectation: virus expression
Co-suppression of transgene and virus particles via RNA.

Hamilton and Baulcombe 1998

Identification of short antisense RNA sequences
dsRNA?
How?

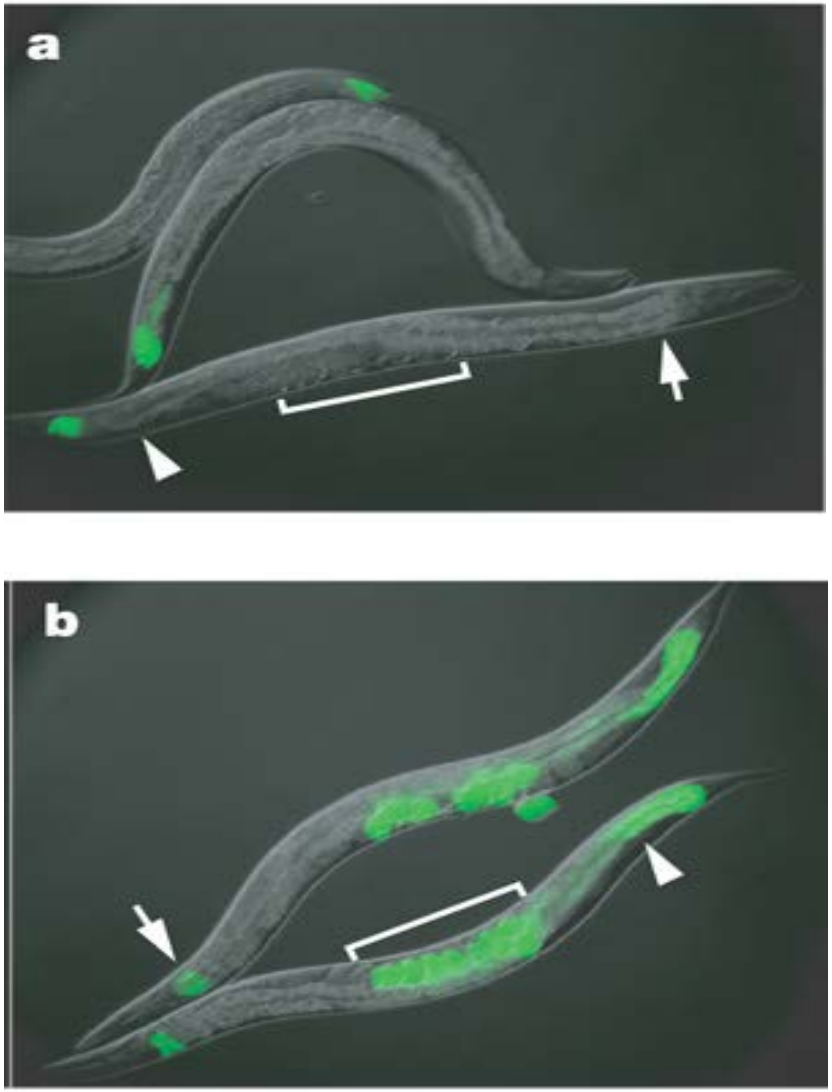
Fire and Mello 1998

Injection of dsRNA into C. elegans
RNA interference (RNAi) or silencing

Ambros 1993 (2000)

Identification of small RNA in C. elegans (micro RNA)

- RNAi in *C. elegans*
  - Silencing of a green fluorescent protein (GFP) reporter in *C. elegans* occurs when animals feed on bacteria expressing GFP dsRNA (**a**) but not in animals that are defective for RNAi (**b**).
    - Note that silencing occurs throughout the body of the animal, with the exception of a few cells in the tail that express some residual GFP.
    - The lack of GFP-positive embryos in **a** (bracketed region) demonstrates the systemic spread and inheritance of silencing.



Called co-suppression because suppressed expression of both endogenous gene and transgene.

Two mechanisms can explain this transgene-mediated gene silencing

Transcriptional gene silencing

Post Transcriptional Gene Silencing (PTGS)
mRNA is made, but then degraded

In 1995 Guo and Kemphues wanted to show that they had cloned the *C. elegans par-1* gene (required for normal division of the zygote). Used antisense RNA to prove.

par-1

Antisense *par-1*

Injection produced mutant *par-1* phenotype

Sense *par-1*control

Injection produced mutant *par-1* phenotype

What?

What is RNA interference?

--Gene “knockdown”

--A cellular mechanism that degrades unwanted RNAs in the cytoplasm but not in the nucleus. Why?

--A way for the cell to defend itself.

What is RNAi?

- RNA interference (RNAi) is an evolutionally highly conserved process of post-transcriptional gene silencing (PTGS) by which double stranded RNA (dsRNA) causes sequence-specific degradation of mRNA sequences.
- It was first discovered in 1998 by Andrew Fire and Craig Mello in the nematode worm *Caenorhabditis elegans* and later found in a wide variety of organisms, including mammals.

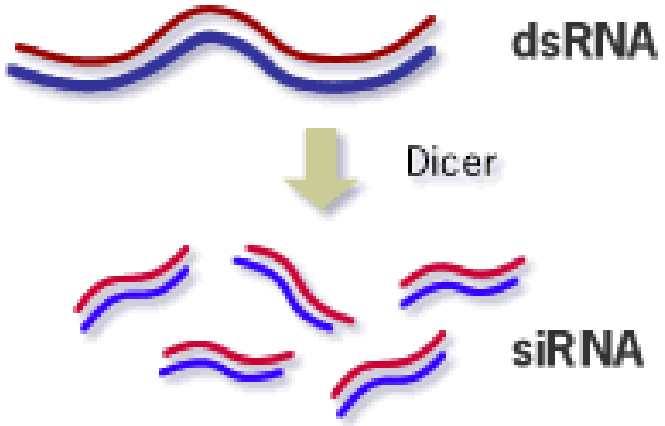
RNAi is a conserved mechanism

- RNAi is a universal, omnipresent conserved mechanism in eukaryotic cells.

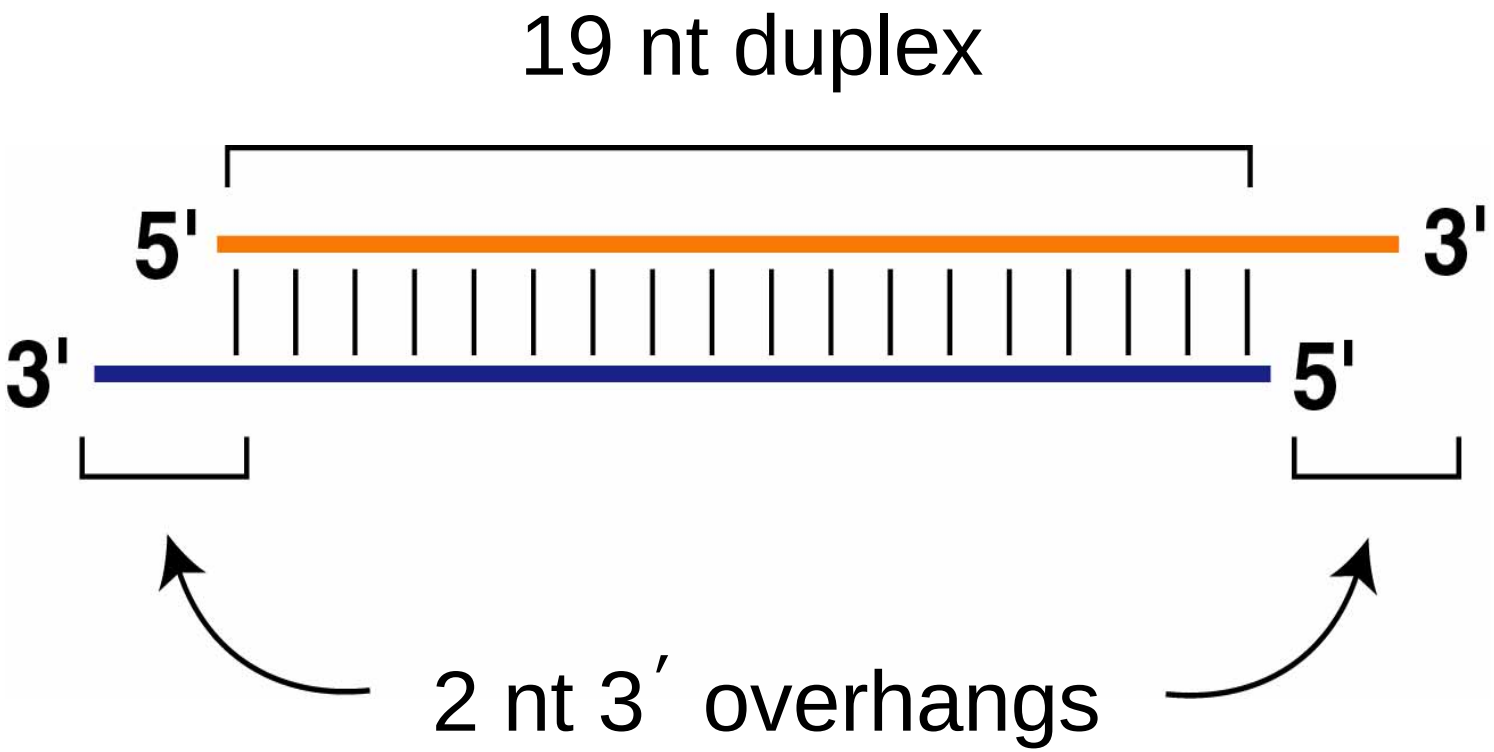
- key proteins involved in RNAi in disparate organisms are highly conserved.

Step 1

- dsRNA is processed into sense and antisense RNAs
  - 21-25 nucleotides in length
  - have 2-3 nt 3' overhanging ends
  - Done by *Dicer* (an RNase III-type enzyme)

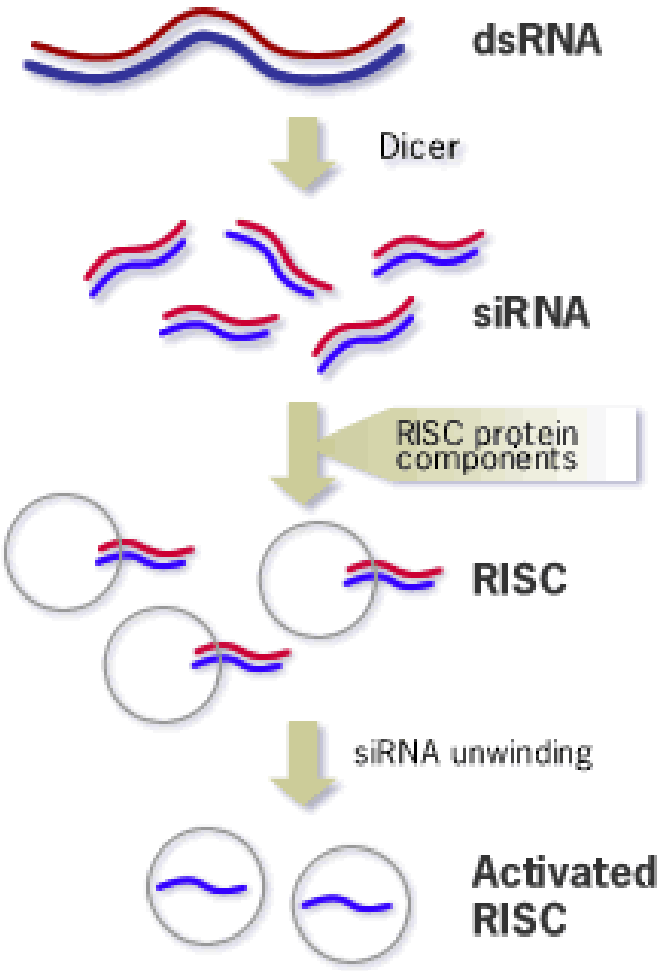


siRNAs have a defined structure



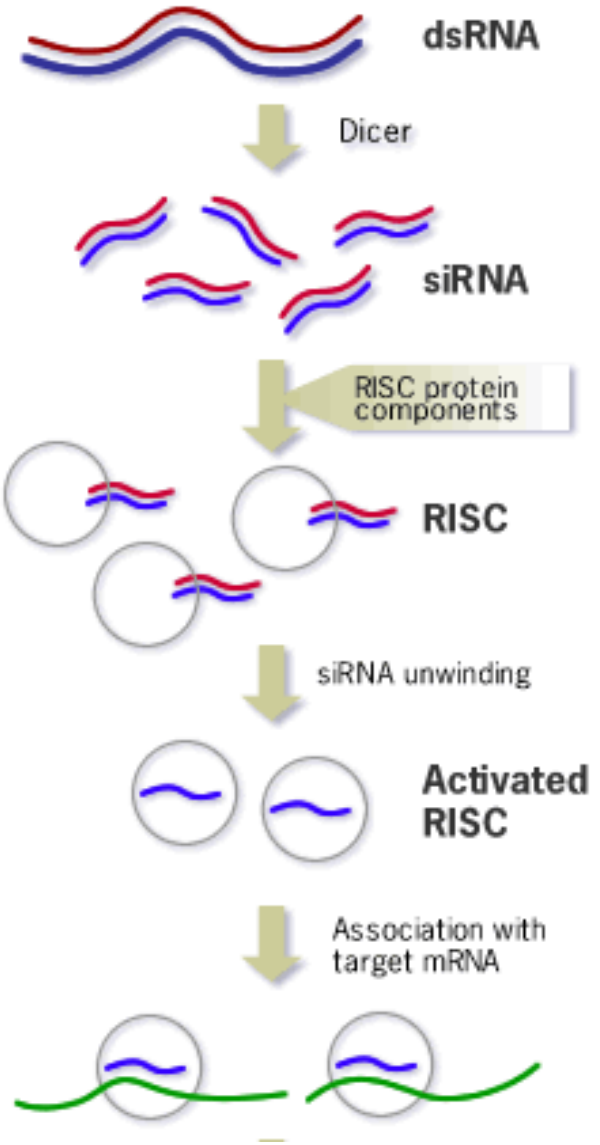
Step 2

- The siRNAs associate with *RISC* (RNA-induced silencing complex) and unwind



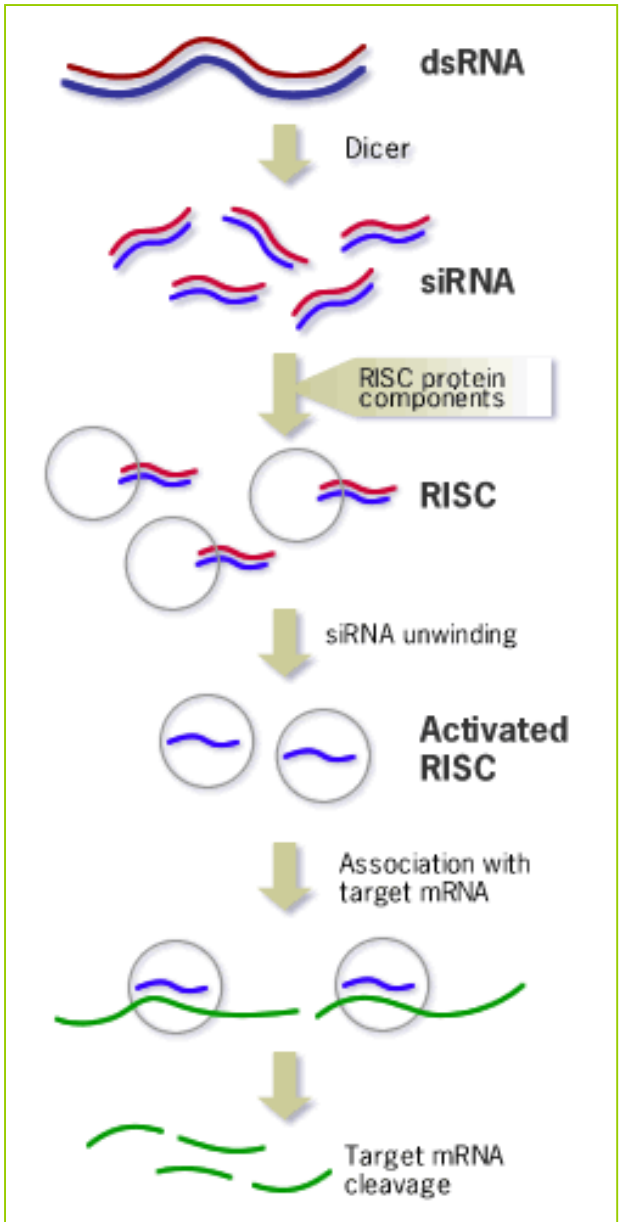
Step 3

- the antisense siRNAs act as guides for *RISC* to associate with complimentary single-stranded mRNAs.

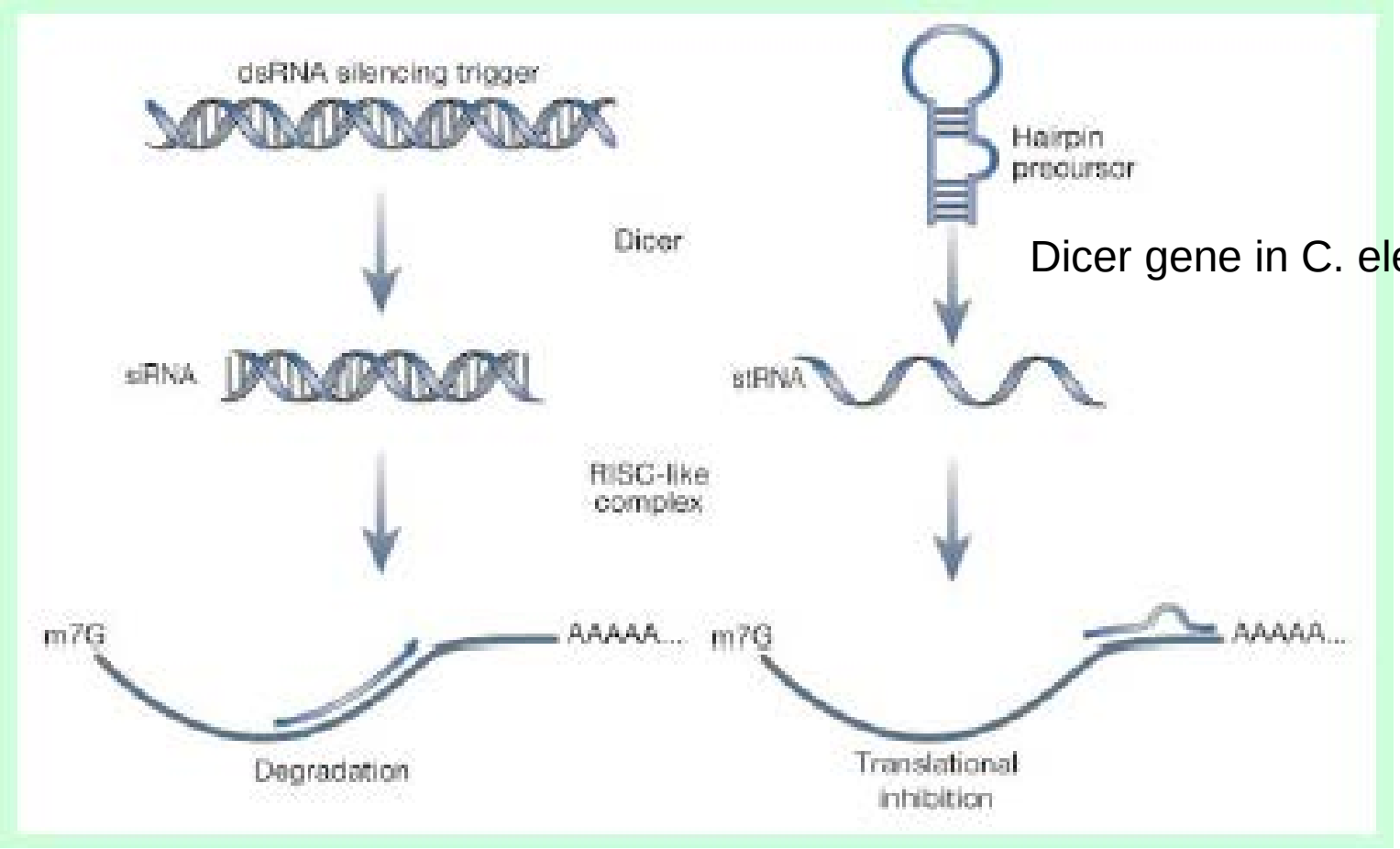


Step 4

- *RISC* cuts the mRNA approximately in the middle of the region paired with the siRNA
- The mRNA is degraded further



Gene regulation by small RNAs

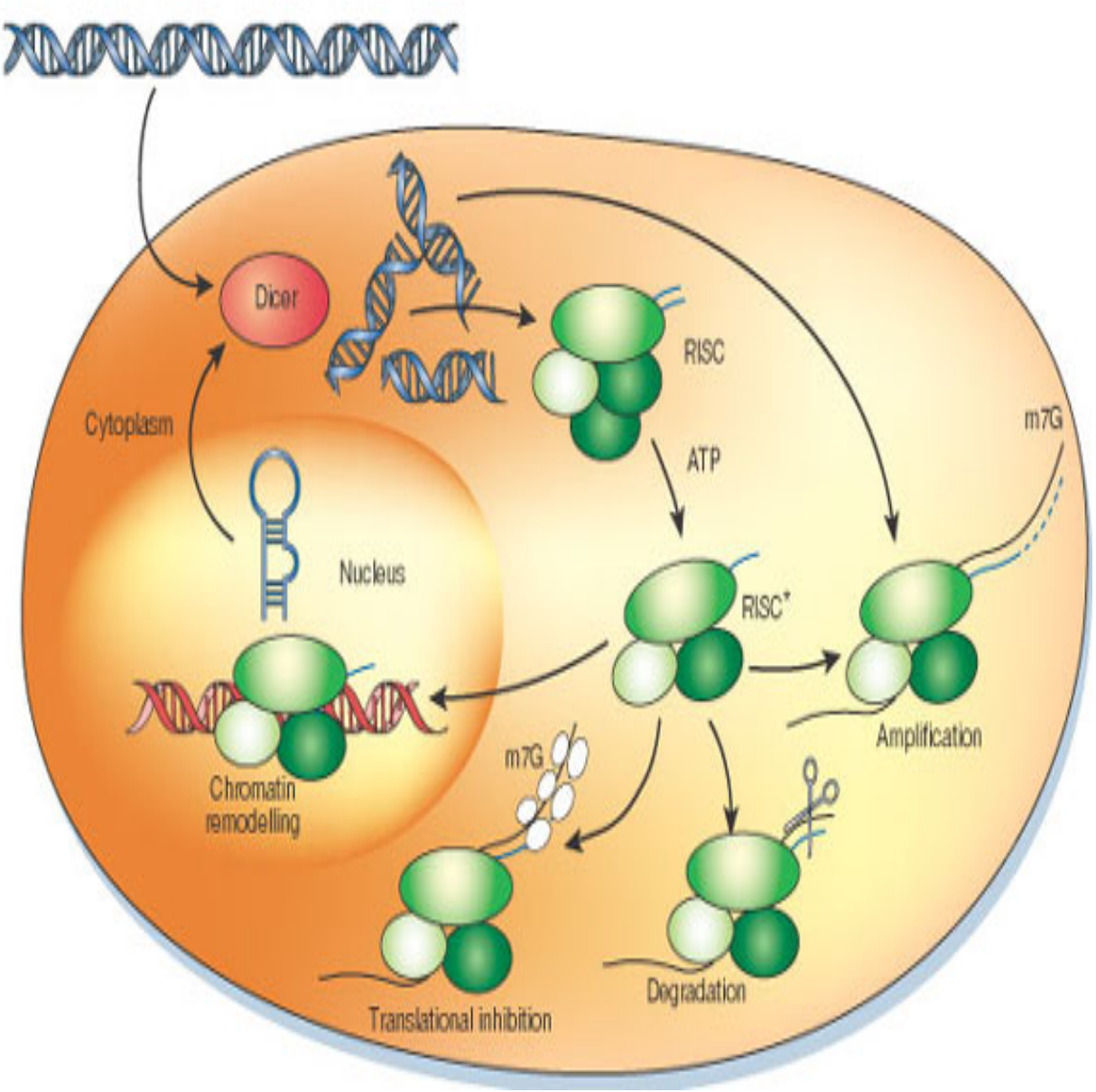


Dicer gene in C. elegans

siRNAs degrade mRNA to stop gene expression quickly

Small temporal(St) RNAs prevent translation to stop gene expression quickly

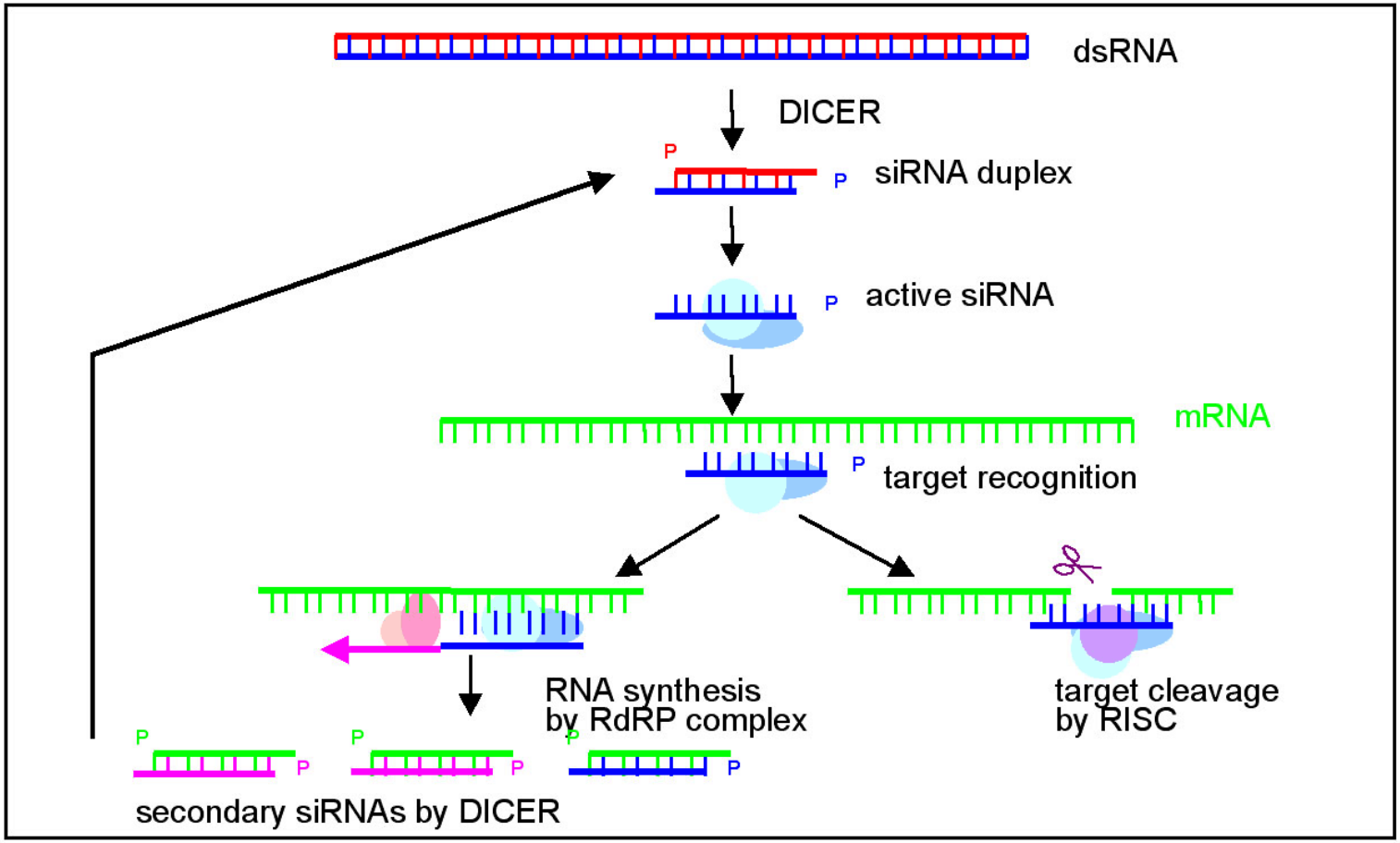
- **A model for the mechanism of RNAi**
  - Silencing triggers in the form of double-stranded RNA may be presented in the cell as synthetic RNAs, replicating viruses or may be transcribed from nuclear genes.
  - These are recognized and processed into small interfering RNAs by **Dicer**.
  - The duplex siRNAs are passed to **RISC** (RNA-induced silencing complex)
  - The complex becomes activated by unwinding of the duplex.
  - Activated RISC complexes can regulate gene expression at many levels:
    - promoting RNA degradation
    - translational inhibition
    - chromatin remodelling
  - Amplification of the silencing signal in plants may be accomplished by siRNAs priming RNA-



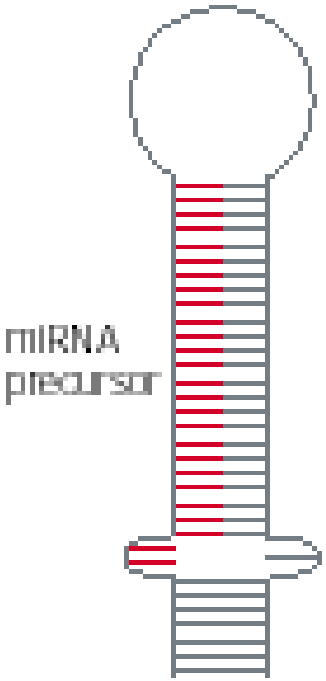
RNAi Amplification

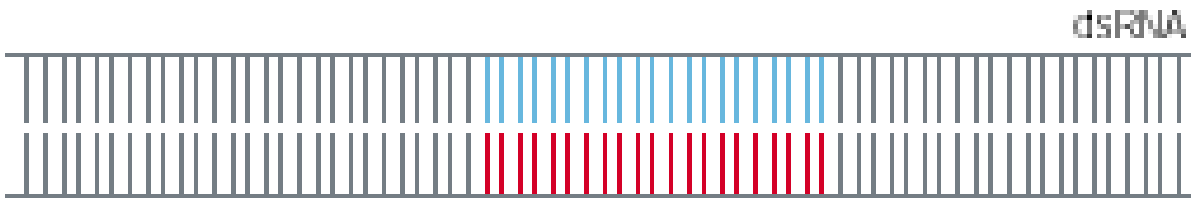
- A small amount of dsRNA can silence a vast amount of target mRNA in *C. elegans*.
- Mechanistic explanations for this observations:
  - Catalytic mechanism: each siRNA fragment can be used several times.
  - **RNA directed RNA synthesis**

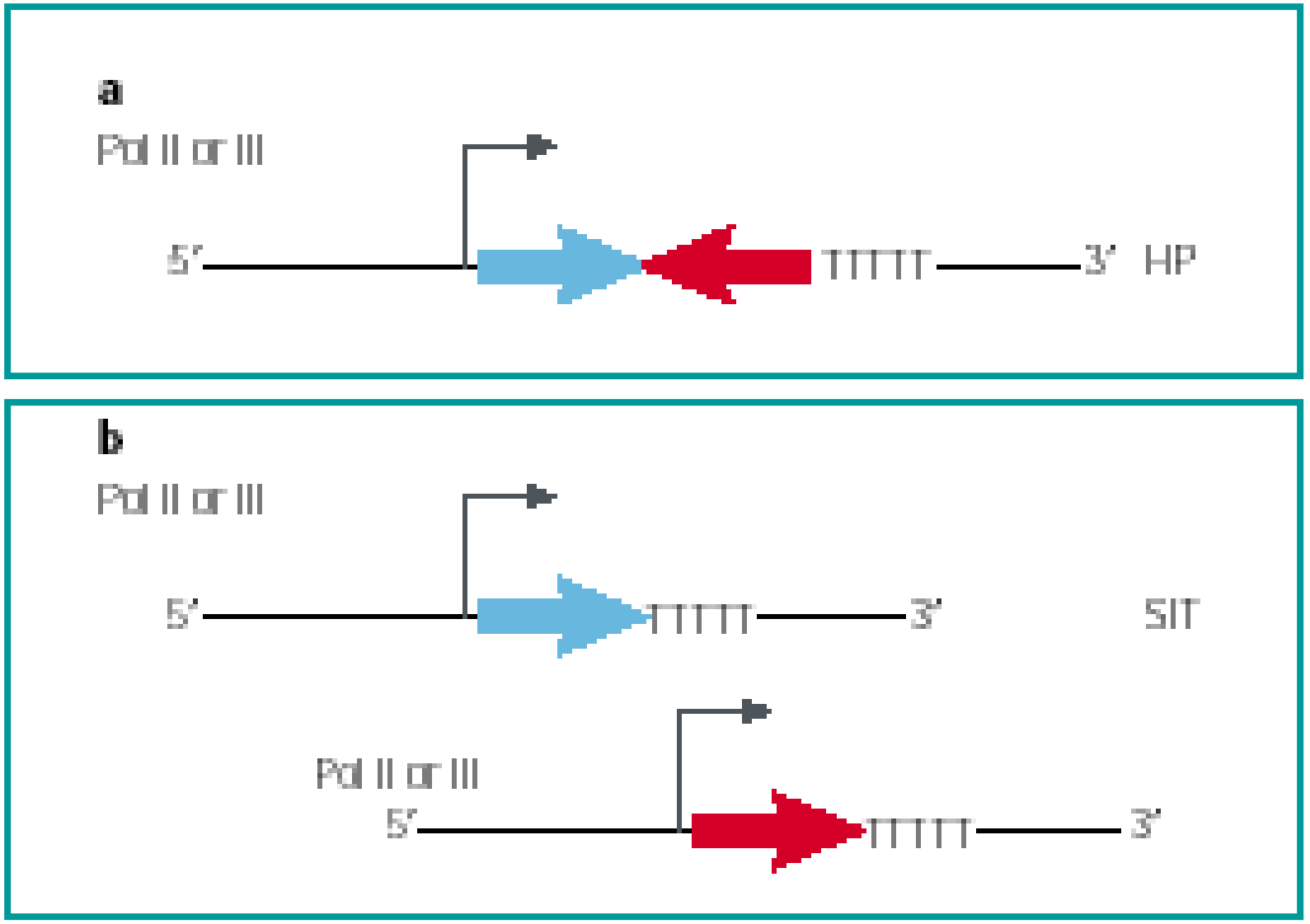
- RNA Dependent RNA Polymerase (RdRP)
  - RdRP activity found in plants and *C. elegans*
  - siRNA acts as primer for elongation on target mRNA

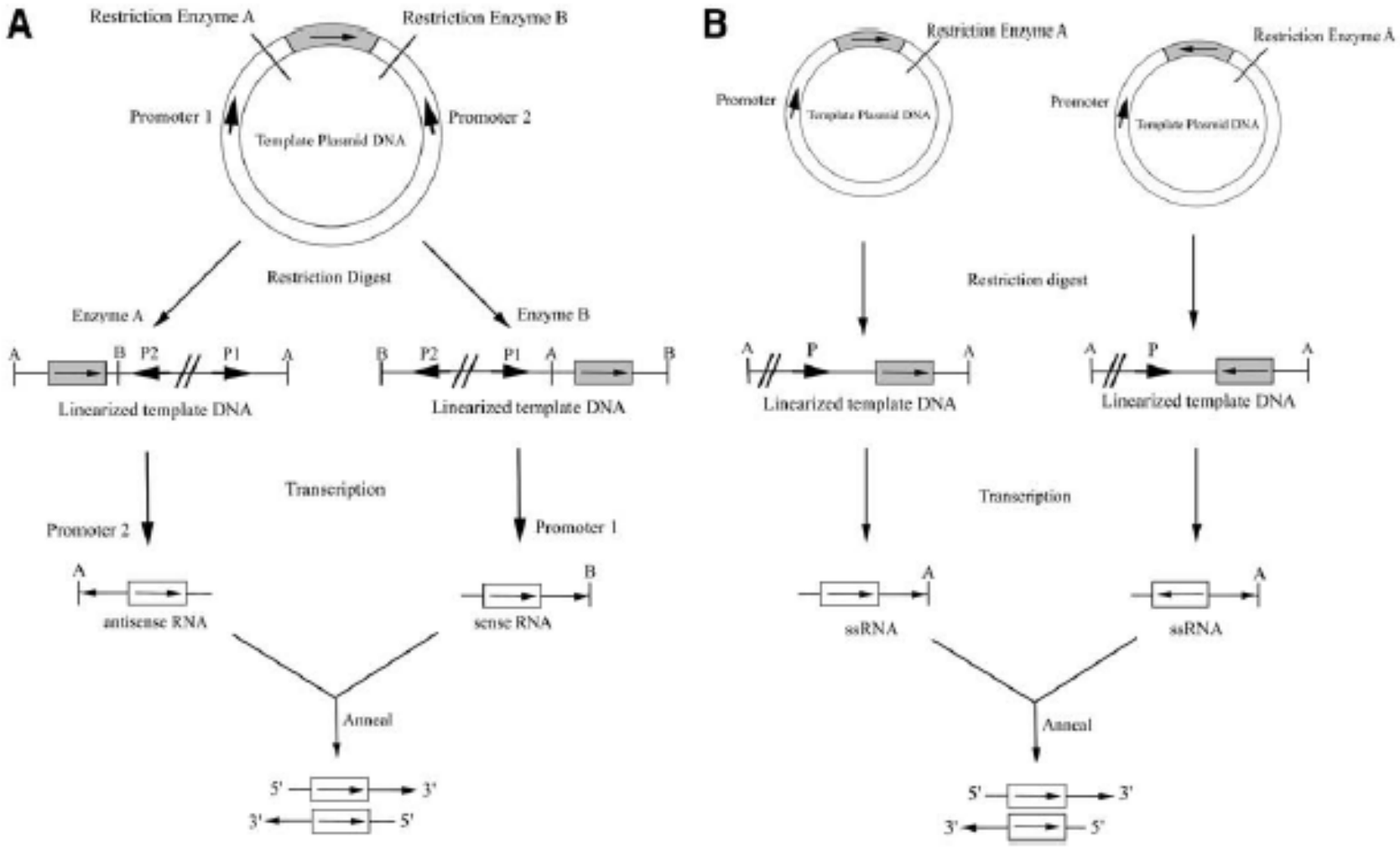


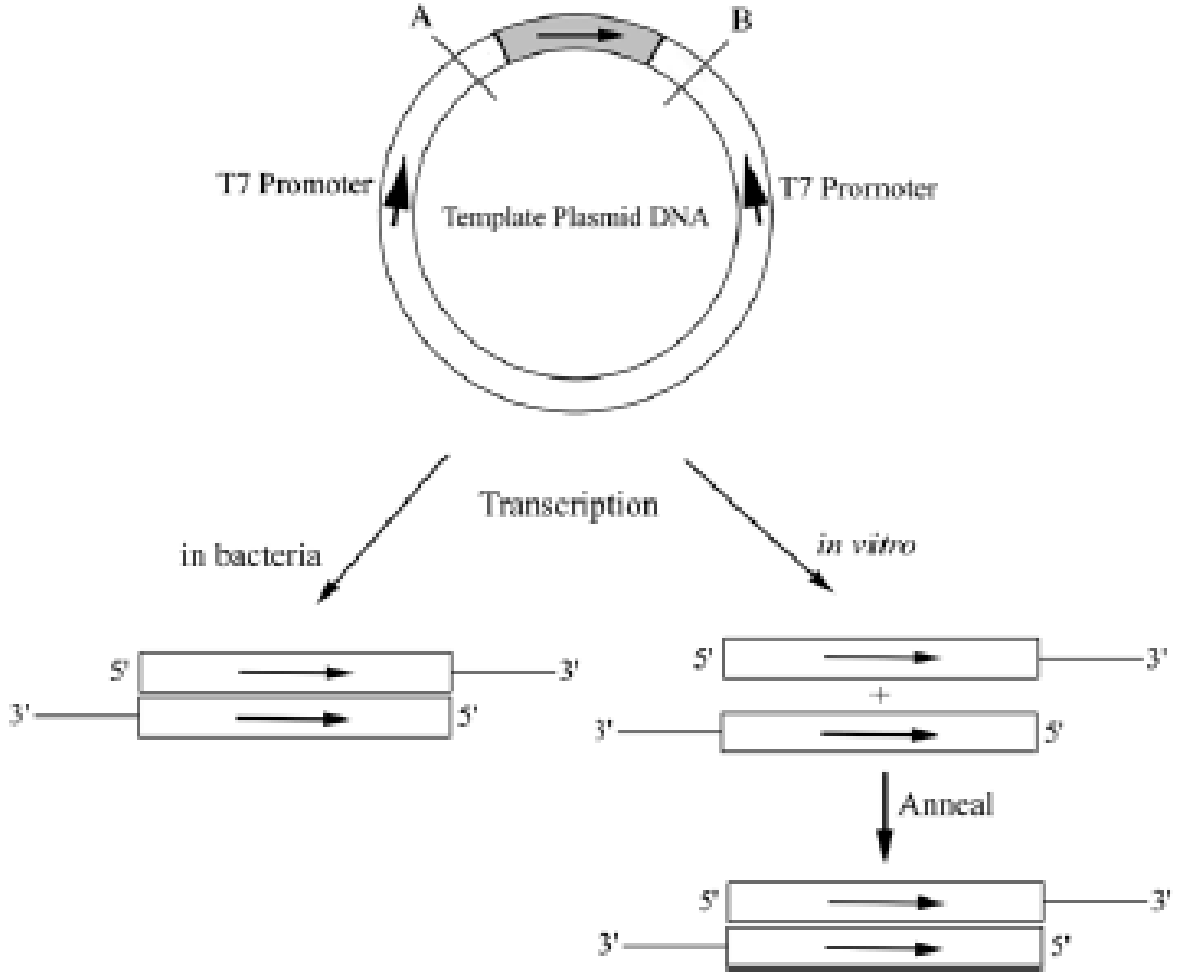
Two ways to get double stranded RNA

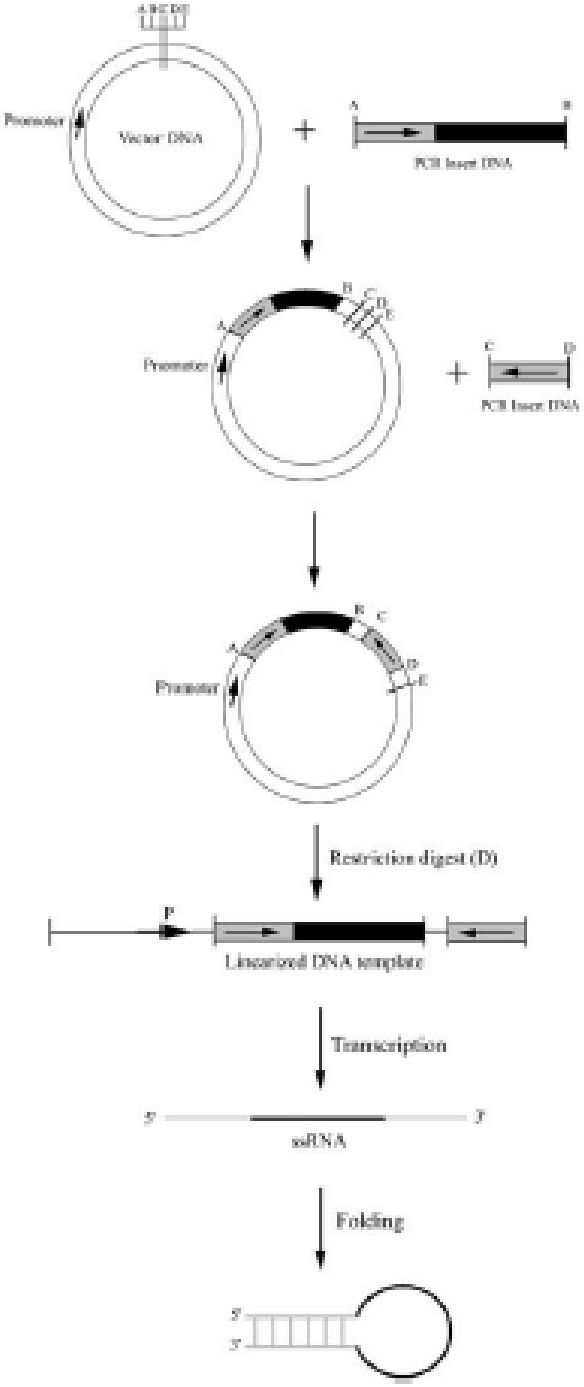




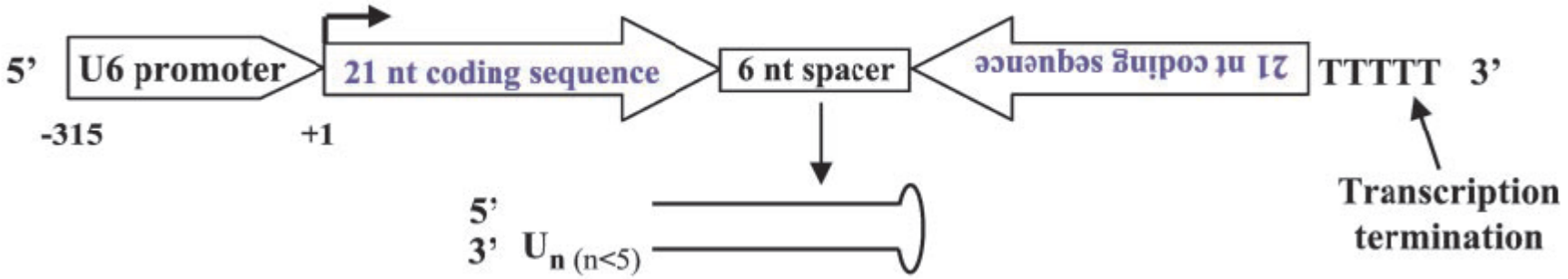

a
Pol II or III
5′
TTTTT
3′
HP
b
Pol II or III
5′
TTTTT
3′
SIT
Pol II or III
5′
TTTTT
3′


A
Restriction Enzyme A
Restriction Enzyme B
Promoter 1
Promoter 2
Template Plasmid DNA
Restriction Digest
Enzyme A
Enzyme B
A
B
P2
P1
A
B
P2
P1
A
B
Linearized template DNA
Linearized template DNA
Transcription
Promoter 2
Promoter 1
A
antisense RNA
B
sense RNA
Anneal
5'
3'
3'
5'
B
Restriction Enzyme A
Restriction Enzyme A
Promoter
Promoter
Template Plasmid DNA
Template Plasmid DNA
Restriction digest
A
P
A
A
P
A
Linearized template DNA
Linearized template DNA
Transcription
A
A
ssRNA
ssRNA
Anneal
5'
3'
3'
5'

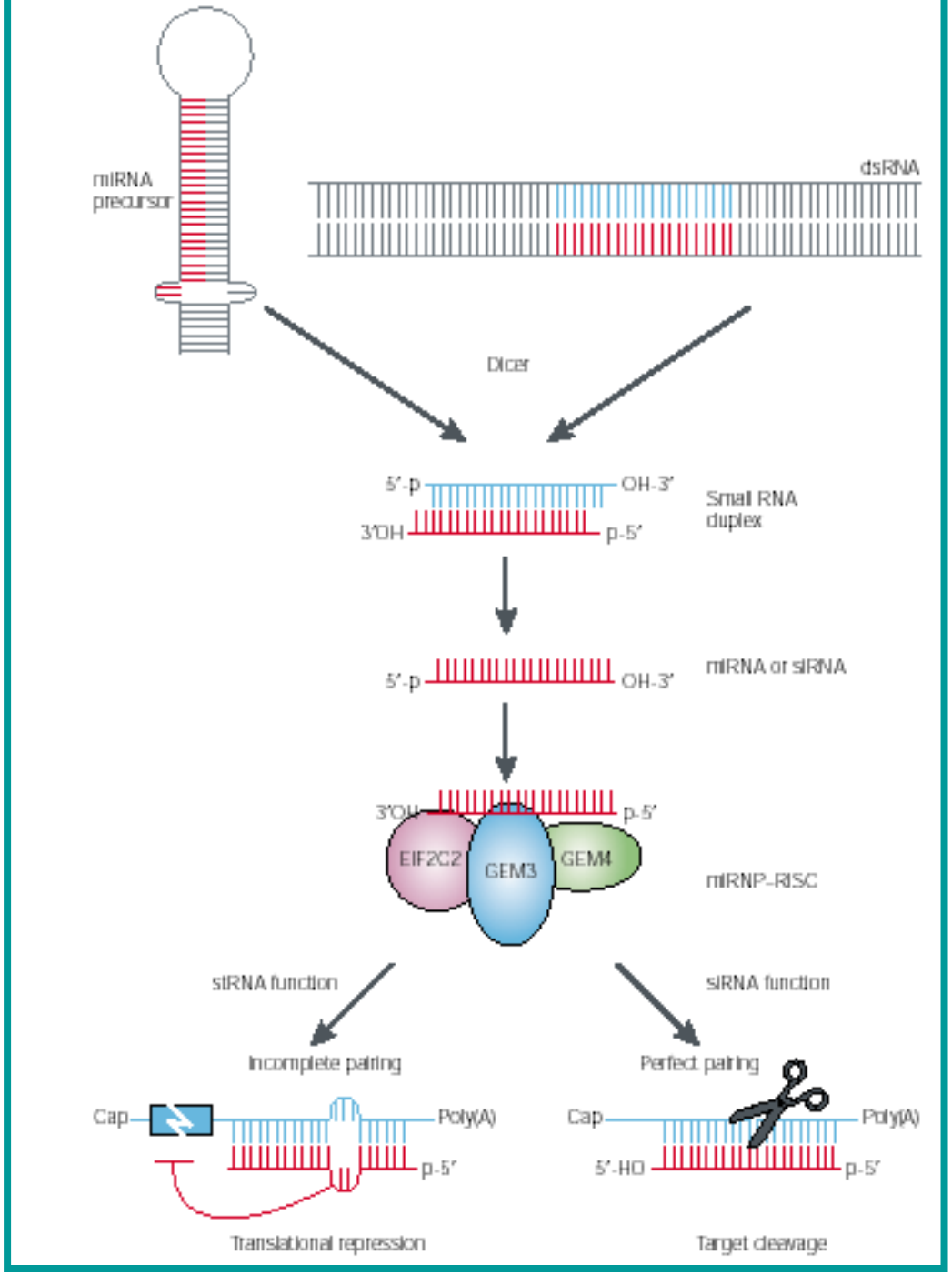

A
B
T7 Promoter
Template Plasmid DNA
T7 Promoter
Transcription
in bacteria
in vitro
5'
3'
3'
5'
5'
3'
+
3'
5'
Anneal
5'
3'
3'
5'


A B C D E
Promoter
Vector DNA
A
B
PCR Insert DNA
B
C
D
E
Promoter
C
D
PCR Insert DNA
B
C
Promoter
D
E
Restriction digest (D)
P
Linearized DNA template
Transcription
5'
3'
ssRNA
Folding
5'
3'

construct for siRNAs



Predicted CD8 Stem Loop


miRNA
precursor
dsRNA
Dicer
5′-p
OH-3′
3′OH
p-5′
Small RNA
duplex
5′-p
OH-3′
miRNA or siRNA
3′OH
p-5′
EIF2C2
GEM3
GEM4
miRNP–RISC
stRNA function
siRNA function
Incomplete pairing
Perfect pairing
Cap
Poly(A)
p-5′
Cap
Poly(A)
5′-HO
p-5′
Translational repression
Target cleavage

Practical Aspects of RNAi

- biological research
  - defining gene function (gene knockout)
    - *C. elegans* genome RNAi projects
  - defining biochemical pathways
    - microarray screening of RNAi knockouts

- therapeutic treatment
  - cancer
  - viral infection
  - parasitic infection