# Gene Silencing

Gene Silencing-based Disease Resistance

Mechanisms And Roles Of The RNA-based Gene Silencing

A Model Of RNA Silencing: Transcriptional Gene Silencing & Post-transcriptional Gene Silencing (PTGS)

RNA interference (RNAi)

# Gene Silencing

- **Gene silencing** was coined to satisfy the need for a term encompassing different gene inactivation phenomena while they were being discovered.
  - Originally, the term **gene suppression** was used to describe transcriptional transgene inactivation in plants (1989).
- **Gene Silencing** is the suppression of gene expression via a variety of methods (e.g., via RNA interference (RNAi), chemical genetics, effect of certain viruses, "zinc finger proteins", sense or antisense genes, etc.).
- **Gene silencing-based disease resistance** can be best studied in mutants that either have a defective silencing mechanism or that carry a single gene that has escaped silencing.

# Gene Silencing- **Co-suppression**

- It was observed that transgenes could also become **post-transcriptionally inactivated** and, moreover, transgene inactivation could result in co-suppression of homologous endogenous genes
  - **Co-suppression** is a significant decrease ("silencing") in the expression of a gene (within an organism's genome/DNA) that (often) results when man inserts and causes to be expressed a homologous gene.
    - For example, **high-oleic oil soybeans** result when the **GmFad2-1** gene (which codes for native D12 desaturase enzyme) is inserted and expressed in traditional varieties of soybeans.
    - That is because the inserted gene "silences" itself and the endogenous **GmFad2-1 gene,** which thus prevents formation of the *D12 desaturase enzyme* (which normally causes most oleic acid within soybeans to be converted into polyunsaturated linoleic acid).

# Gene Silencing- HDGS

- Further examples of both, transcriptional and post-transcriptional transgene inactivation pointed to a dependence of gene inactivation processes on the existence of homologous sequences leading to the term **homology-dependent gene silencing** (HDGS)
  - **HDGS** was believed to be a result of DNA–DNA interactions,

# Gene Silencing- **VIGS**

- However, it was recognized that single copy genes also became silenced and this demonstrated that DNA– DNA interactions were not an absolute requirement
  - These findings were substantiated by the phenomena of **RNA-mediated virus resistance** and **virus induced gene silencing** (VIGS) .
  - Instead of the mRNA of an endogenous gene, the viral RNA becomes a target for degradation.
- The difference between **co-suppression** and **RNA-mediated virus resistance** is that the latter process can result without any involvement of virus-specific DNA.
  - VIGS was also shown to proceed without any DNA–DNA interactions.

# Gene Silencing- **dsRNA**

- RNA viruses that do not replicate via DNA intermediates were capable of inducing (trans)gene silencing.
  - Homology between the replicating viral RNA and the (trans)gene mRNA was sufficient to initiate the silencing process.
- A breakthrough in the understanding of these observations came from **silencing experiments** in the nematode *Caenorhabditis elegans*.
  - Injection of double-stranded RNA (dsRNA) into worm cells triggered specific degradation of homologous (trans)gene mRNA.

# Gene Silencing- **RNAi**

- Similar experiments demonstrated that this **RNA interference (RNAi)** mechanism functioned in a variety of organisms including humans.
  - RNA Interference (RNAi) Coined by Andrew Fire and Craig Mello in 1998, this term refers to what happens when short strands of (complementary) double-stranded RNA (dsRNA) are introduced into living cells.
- That interaction can be done either by
  - physical insertion of the dsRNA
  - or by genetic engineering of the organism
- so the organism's cell(s) themselves produce that (new) dsRNA
  - For example, genetic engineers can utilize T7 RNA polymerase to cause the production of such dsRNA within living cells.
- Thus, viral infection (i.e., insertion of viral dsRNA) and also **micro-RNAs** can also cause RNA interference.
  - ***dsRNA) as a trigger or intermediate***.

# Gene Silencing-based Disease

- In numerous organisms, a variety of severe diseases are caused by the attack of invasive nucleic acids (INA) such as:
  - Viruses and retroviral
  - Transposable elements (transposon):
    - Transposon is a DNA sequence able to replicate and insert one copy (of itself ) at a new location in the genome.
      - Transposon was discovered in 1950 by geneticist Barbara McClintock in corn plants (maize) (Zea mays L.); and in bacteria a decade later by Joshua Lederberg.
    - Transposons can either carry genes along one organism's genome, or even into another organism's genome (e.g., via sexual conjugation, in bacteria).
      - By such sexual conjugation, transposons can carry genes that confer new phenotypic properties (e.g., resistance to certain

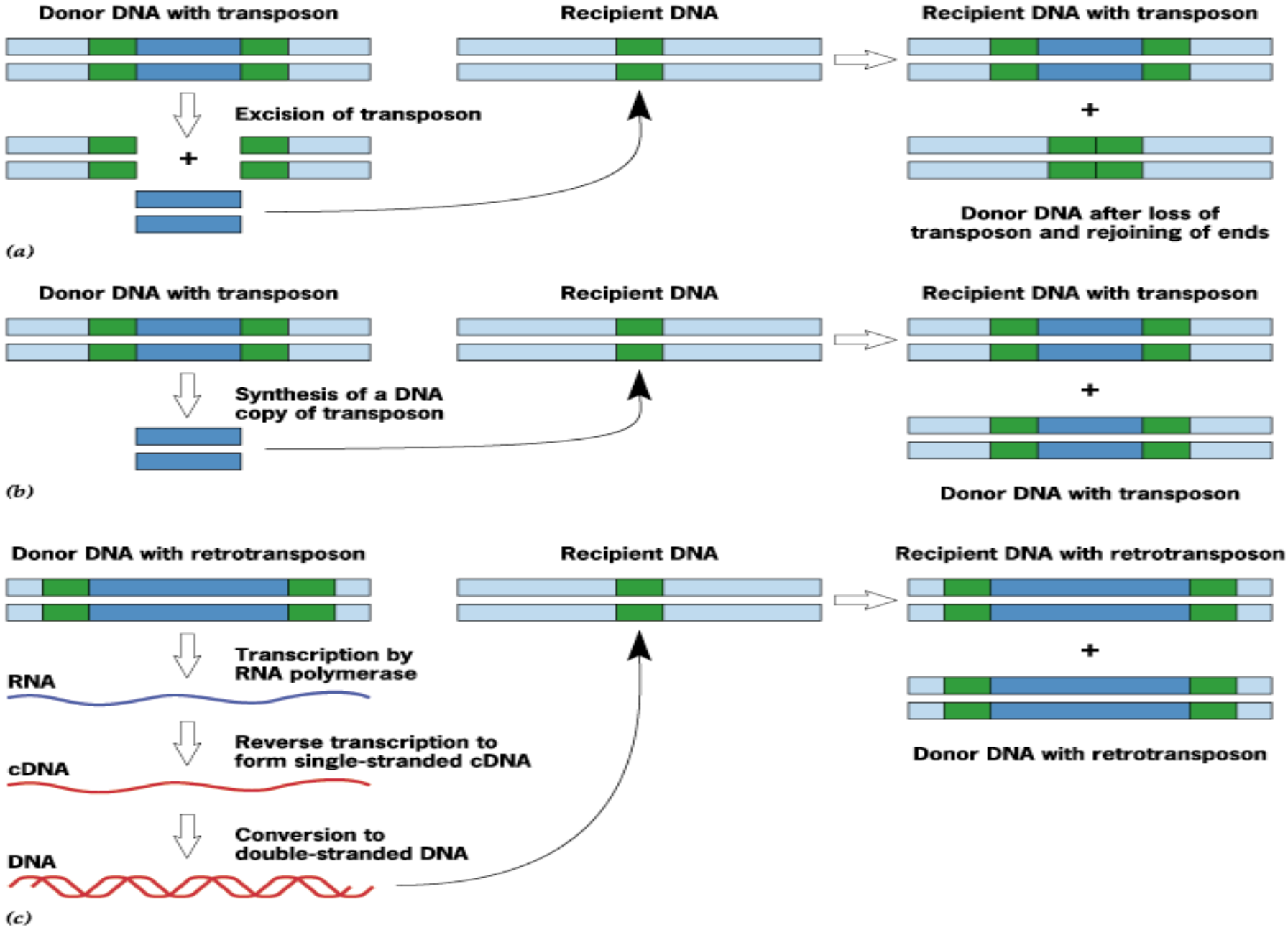



**Figure 10.30** Three alternate pathways by which transposable elements move from place to place within the genime.

# Gene Silencing-based Disease **Resistance**

- Because of the difficulties in giving disease a precise definition, gene silencing-based disease resistance will be restricted to the description of gene inactivation processes that contribute to maintain the physical fitness of an organism.
  - In this sense, we then are concerned with the elimination of invasive nucleic acid expressing.
  - Fungi, plants, invertebrates and vertebrates also enlist gene silencing systems to counteract the harmful effects of invasive nucleic acids.
  - In particular, plants that lack interferon and immune responses have established efficient **transcriptional** and **post-transcriptional gene silencing systems.**

# **Gene Silencing-** In plants:

- RNA silencing was first discovered in transgenic plants where it was termed **co-suppression or post-transcriptional gene silencing (PTGS)**.

- The experiments were designed to examine whether dsRNA is also capable of triggering (trans)gene inactivation.
  - It was demonstrated that dsRNA was an efficient inducer of post-transcriptional gene silencing (PTGS) pointing to a relationship between RNAi and PTGS.
  - In addition to experiments illustrating the significance of dsRNA in PTGS, the potential of dsRNA to initiate transcriptional gene silencing (TGS) was also shown in plant systems.
  - Expression or introduction of **dsRNA sharing homology with promoter sequences** led to specific inhibition of transcription of the corresponding genes.
    - Such gene inactivation phenomena are now referred to as **RNA silencing**.

# A model of RNA Silencing

- There is **sophisticated machinery** for homology-dependent gene silencing (**HDGS**) that seems essentially common between algae, fungi, plants and animals.

- At least four entirely independent lines of research led to this realization:
  - Transgene-dependent gene silencing in plants (co-suppression)
  - RNA interference (RNAi) in diverse animals
  - Quelling in fungi
  - Transposable elements silencing

## Transgene-dependent Gene Silencing In Plants (**Co-suppression**)

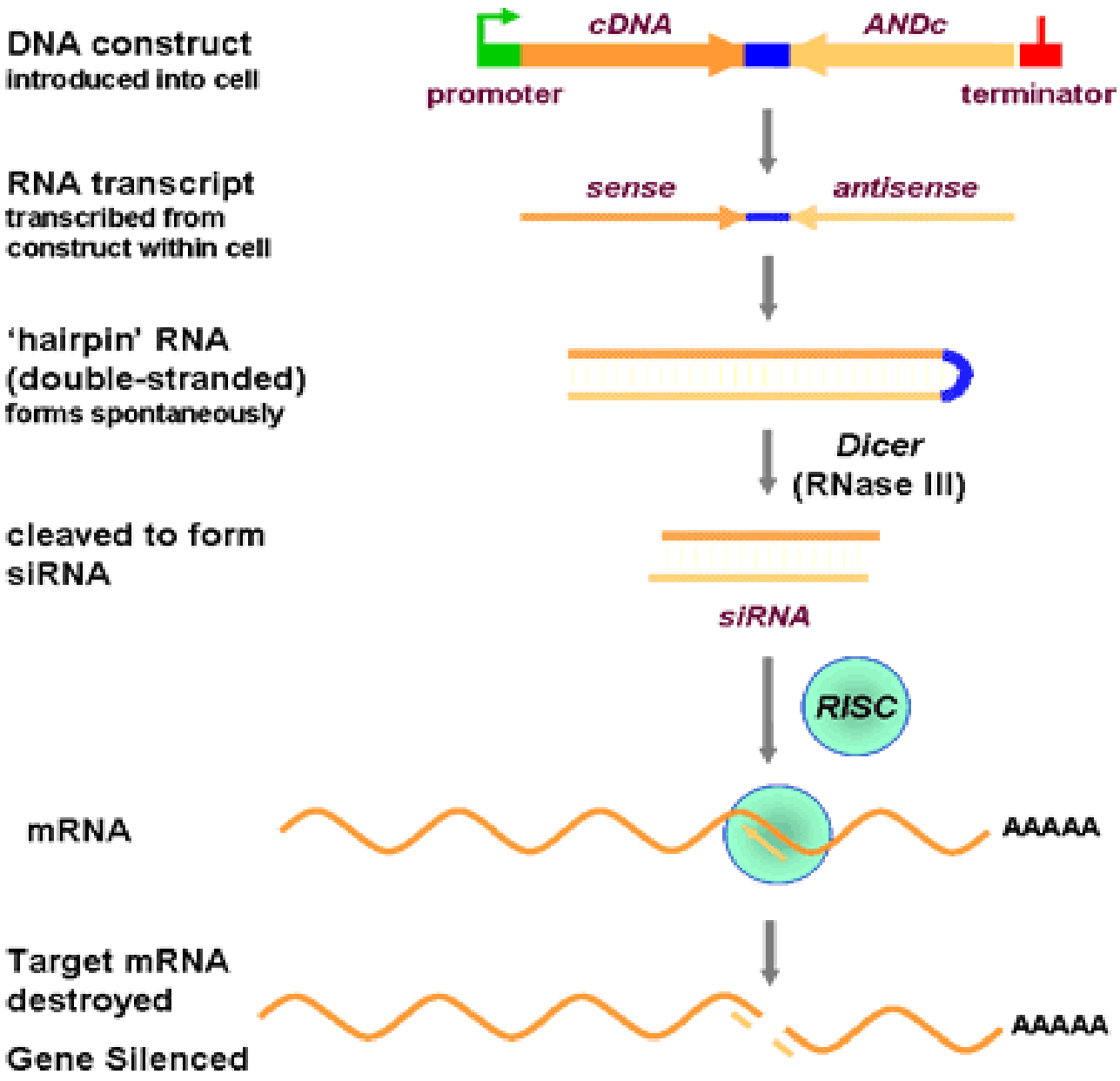
- Now we can distinguish two distinct types of **homology-dependent gene silencing** (HDGS) phenomena are observed in plants:

1. **Transcriptional gene silencing (TGS).**
2. **Post transcriptional gene silencing (PTGS).**

# Transcriptional gene silencing (TGS )

- TGS may be triggered directly by :
  1. **Methylation of homologous promoter** regions in the genome.
  2. Transcription of **Inverted DNA Repeat** Sequences in the nucleus

- **RNA-directed DNA Methylation** (RdDM) and **post-transcriptional gene silencing/RNA interference** (PTGS/RNAi) are both **triggered by dsRNAs**


DNA construct
introduced into cell
cDNA
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promoter
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RNA transcript
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'hairpin' RNA
(double-stranded)
forms spontaneously
Dicer
(RNase III)
cleaved to form
siRNA
siRNA
RISC
mRNA
AAAAA
Target mRNA
destroyed
AAAAA
Gene Silenced

# Transcriptional gene silencing

- DsRNAs can be made by:

1. transcribing through **inverted DNA repeats (IR)**

2. the activity of cellular RNA-dependent RNA polymerase **(cRdRP) acting on '<u>aberrant</u>' RNA** templates synthesized from <u>single copy (SC)</u> genes in the nucleus or generated in the cytoplasm by **RISC** cleavage of mRNA
   - '**<u>aberrant</u>**' (prematurely terminated and / or lacking polyadenylation)
3. **Replicating RNA viruses** produce dsRNA by means of a viral RdRP (vRdRP).

# Cellular protein involved in gene silencing

- Proteins with homology to RNA-directed RNA polymerases function in post-transcriptional gene silencing in :
  - Fungus *neurospora crassa*, (Quelling)
  - Nematode *caenorhabditis elegans*, (RNAi )
  - Mustard plant *arabidopsis thaliana (C*o-suppression)
- These findings are consistent with a conserved mechanism operating in these diverse species.

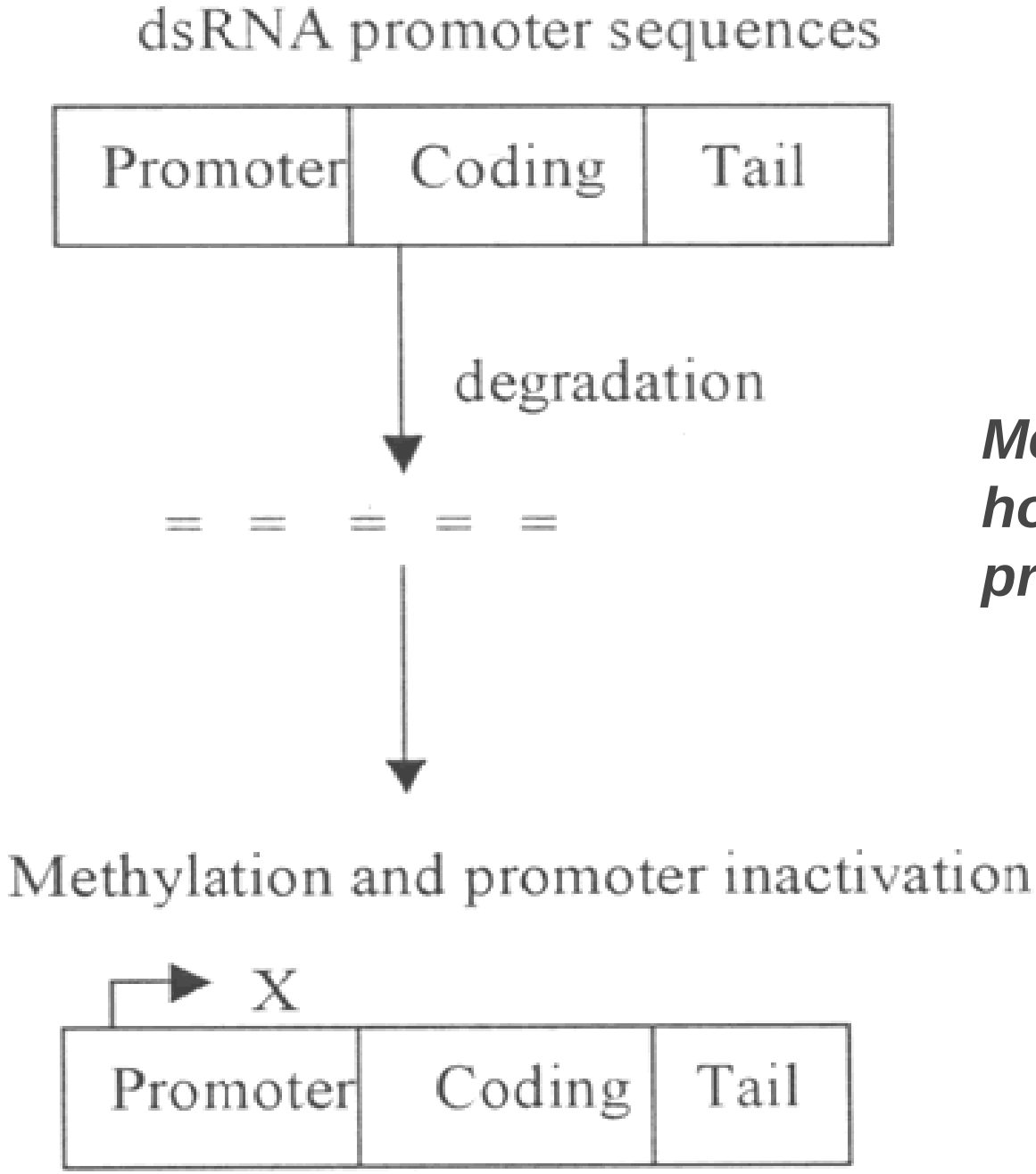
**Conserved proteins involved in PTGS**

| Protein family | *N. crassa* | *C. elegans* | *A. thaliana* |
|---|---|---|---|
| RdRP | QDE-1 | EGO-1 | SGS2/SDE1 |
| Piwi/Sting | QDE-2 | RDE-1 | ? |
| WRN protein | QDE-3 | MUT-7 | ? |

Gene names in full: EGO, enhancer of *glp-1*; MUT, mutator; QDE, quelling defective; RDE, RNAi defective; RdRP, RNA-directed RNA polymerase; SDE, silencing defective; SGS, suppressor of gene silencing; WRN, Werner's syndrome.

# Transcriptional gene silencing

- RNAse III-type enzymes (i.e. **Dicer, Caf**) cleaved dsRNAs into **small interfering RNAs**
  - **siRNAs** is ~ 21-25 nucleotides
- In the cytoplasm,
  - siRNAs serve as guides for endonucleolytic cleavage of homologous mRNA in association with the RNA-induced silencing complex (**RISC**).
  - siRNAs can trigger the degradation of homologous RNAs in the cytoplasm (PTGS)
- In the nucleus;
  - The siRNAs cause *de novo* **methylation** of homologous DNA
  - At the genome level, RNA can induce the epigenetic modification of homologous DNA sequences through the process of RNA-directed DNA methylation (**RdDM**) that has only been demonstrated in plants.




***Methylation of homologous promoter***

# TGS - DNA Methylation

- **DNA Methylation** : Refers to a process resulting in a DNA molecule that is saturated with **methyl gro**ups (i.e., methyl sub-molecule groups --CH3 have attached themselves to the DNA molecule's "backbone" at all possible locations on that DNA molecule).

# DNA Methylation

- DNA methylation is used by healthy cells to:
  - "**turn off**" certain genes when those particular genes are not needed (e.g., they turn-off genes involved in juvenile development after the organism reaches adulthood).
  - Prevent the spread/activation of potentially-harmful nucleic acids (e.g., certain transposable elements) within the organism's genome.
- DNA methylation (of cell genes that would normally prevent inappropriate cell division/proliferation) also occurs in some cancers.

# DNA Methylation

- The four nucleotide bases of DNA [ (C), (A), (G), and (T)] form a total of 16 possible **dinucleotide pai**rs.
- These **CpG dinucleotide** (where a **cytosine** is adjacent to a **guanine** in the 5' direction), occurs at a lower than expected frequency throughout most of the human genome but at a higher than expected frequency in small portions of DNA that are referred to as **CpG islands.**
  - These CpG islands are often concentrated near gene transcription start sites, the promoter regions where the transcription of DNA to RNA begins.
- In the normal cell, most of the CpG dinucleotides at gene promoter regions are unmethylated, whereas CpG islands found at other portions of the genome are generally methylated.

# DNA Methylation

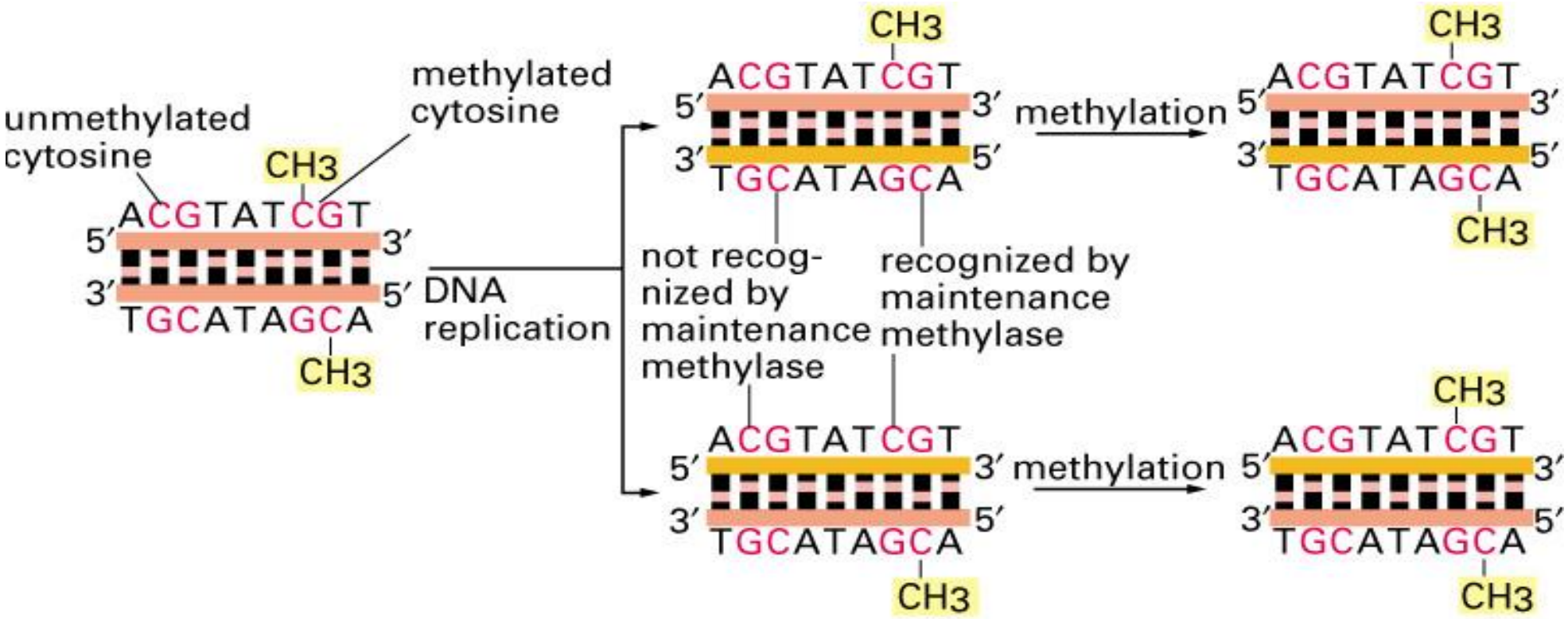



Figure 7–81. Molecular Biology of the Cell, 4th Edition.

# DNA Methylation

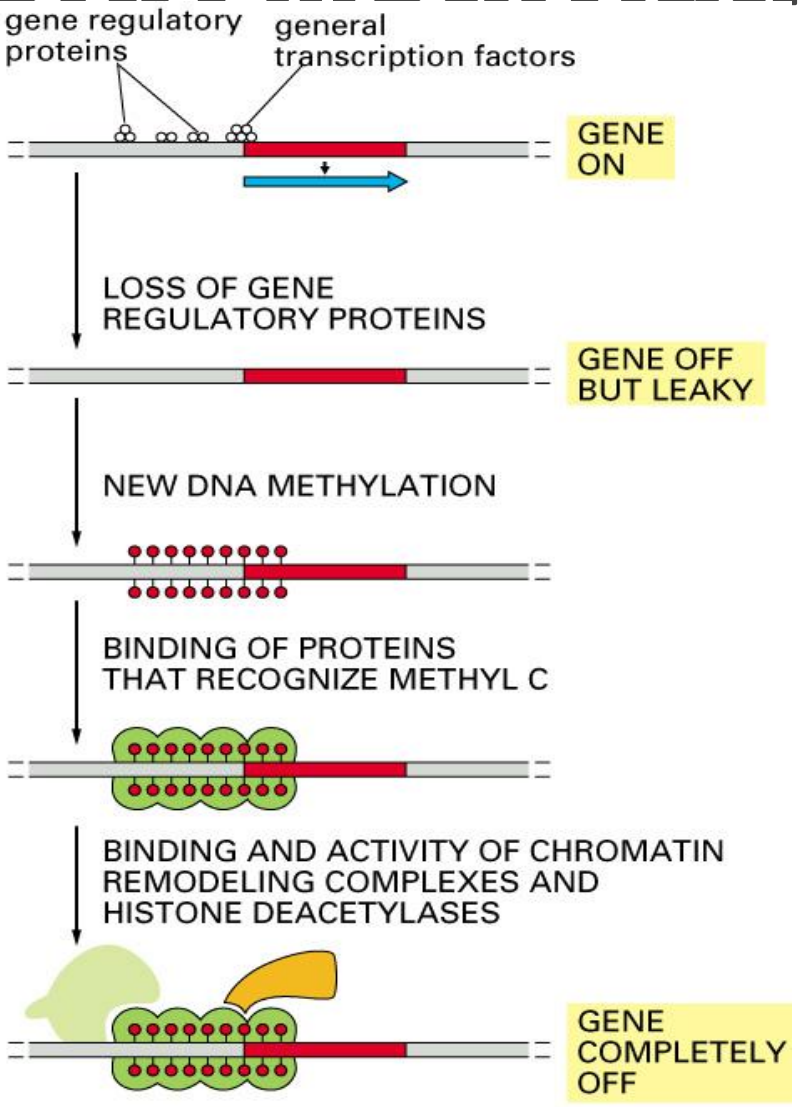
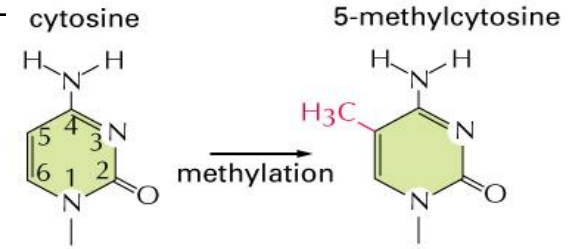



Figure 7–82. Molecular Biology of the Cell, 4th Edition.




- The absence of CpG island methylation is a hallmark of an active transcription site that is capable of transcribing DNA to RNA.

- In cancer cells, this pattern of CpG methylation becomes disrupted:
  - CpG islands in promoter regions of selected genes have an unusually high likelihood of methylation, but CpG dinucleotides that fall outside of promoter regions are less likely than normal to be methylated

# Transcriptional gene silencing

- **RNA-dependent DNA methylation (RdDM**) was first discovered with viroids.

- The ability of viroids and RNA viruses, which produce dsRNA replication intermediates, to trigger RdDM suggested a general requirement for dsRNA in this process

- RdDM results in **dense methylation** at most symmetrical and non-symmetrical **cytosines** within the region of homology between the inducing RNA and the target DNA.

# Transcriptional gene silencing

- A dsRNA transcribed from an IR containing promoter sequences is able to trigger *de novo* methylation and silencing of homologous promoter in trans(gen).

- The promoter dsRNA is degraded to smRNAs, indicating that it is entered the same degradation pathway as dsRNAs involved in PTGS.

# Mechanism of TGS

- The mechanism of RdDM is unknown but is assumed to involved RNA-DNA interactions based on sequence homology.

- RdDM results in dense methylation at cytosines within the region of homology between the inducing RNA and the target DNA. The DNA targets as short as 30bp can be modified.

- **The minimal DNA target size for RdDM is 30 bp**.
  - This opens the possibility that the 21-25 nucleotides (nt) RNA degradation products of dsRNA could be responsible for directing *de novo* methylation

- The smRNAs could conceivably guide the **DNA methyltransferase** to unmodified homologous DNA sequences.

# Mechanism of TGS

- The methylation of promoter sequences usually results in **promoter inactivation**, probably by
  - **histone deacetylation** and **chromatin condensation** (Wassenegger et al. 1994).

- RNA interference is a conserved process in which double-stranded RNA is processed into 21–25 nucleotide siRNAs that trigger **posttranscriptional gene silencing**.

- In addition, plants display a phenomenon termed **RNA-directed DNA methylation (RdDM)** in which DNA with sequence identity to silenced RNA (siRNAs) is ***de novo* methylated** at its **cytosine residues** (Cao et al. 2003).

# Mechanism of TGS

- siRNAs are processed from double-stranded RNA by **Dicer**, an **RNaseIII – RNA helicase**, and their synthesis often requires an ***RNA-dependent RNA polymerase***.

- RNA thus appears to be a general means of targeting *de novo* DNA methylation, which may indicate how **sequence-specific gene silencing** is established in a variety of epigenetic phenomena.

- Several studies **suggest** the possible involvement of **DNA triplet helix structure** in the process of RdDM.
  - These unusual structures might attract ***de novo* DNA methyltransferase** (DNMT). (Tang et al. 2003)

# Mechanism of TGS

- Another possible candidate methyltransferase is the so-called "**chromodomain containing methyltransferases**" [**Chromomethylase (CMT)]**,
  - that has been found only in plants.

- **Chromodomains** are believed to mediate interactions between chromatin regulatory proteins.

- **Small RNAs** might interact with a chromomethylase through the chromodomain to direct methylation of homologous DNA sequences

# Review-HDGS

- At present, RNA has been implicated in two types of **homology-dependent gene silencing** (HDGS):
  1. Posttranscriptional gene silencing (**PTGS**) involves targeted degradation of homologous RNAs in the cytoplasm
  2. **RNA-directed DNA methylation** (RdDM) can be induced by RNA derived from homologous DNA sequence at the genome level.

# Review-HDGS

- **dsRNA** plays a dual role in plant gene silencing by initiating both the RNA-degradation step of PTGS and RdDM.
- Experimental results showed that transcriptional gene silencing (TGS), Posttranscriptional gene silencing (PTGS), and promoter methylation can be triggered by dsRNA
- The mechanisms of these different modes of HDGS and the characteristics of the RNAs involved **are being actively investigated**.

# A model of RNA- based TGS and PTGS

- TGS may be triggered directly by :
  - transcription of **inverted repeat sequences** in the nucleus
  - **methylation of homologous promoter** regions in the genome.

- Some of dsRNA and other aberrant RNAs formed in the nucleus may be transported to the cytoplasm and enter the PTGS pathway.

# A model of RNA- based TGS and PTGS

- Two modes of dsRNA production lead to PTGS in the cytoplasm:
  1. virus induced gene silencing mediated by the **viral RdRP**,
  2. transgene-induced gene silencing mediated by **cellular RdRP**.

- The dsRNA from either of these sources can be targeted by a putative dsRNA specific ribonuclease which generates 21-25 nt RNAs of both polarities (smRNAs).

# Post-transcriptional gene silencing (PTGS)

- That siRNA (i.e., specific to a selected gene's mRNA) causes specific cellular "**cutting enzymes**" in **RISC** (RNA-induced silencing complex) to adhere to the transcribed-from-gene mRNA which the **dsRNA was chosen to be specific to**.

- Those "cutting enzymes" cut-up and mark for destruction the transcribed-from-gene mRNA; thereby negating the effects of that gene.

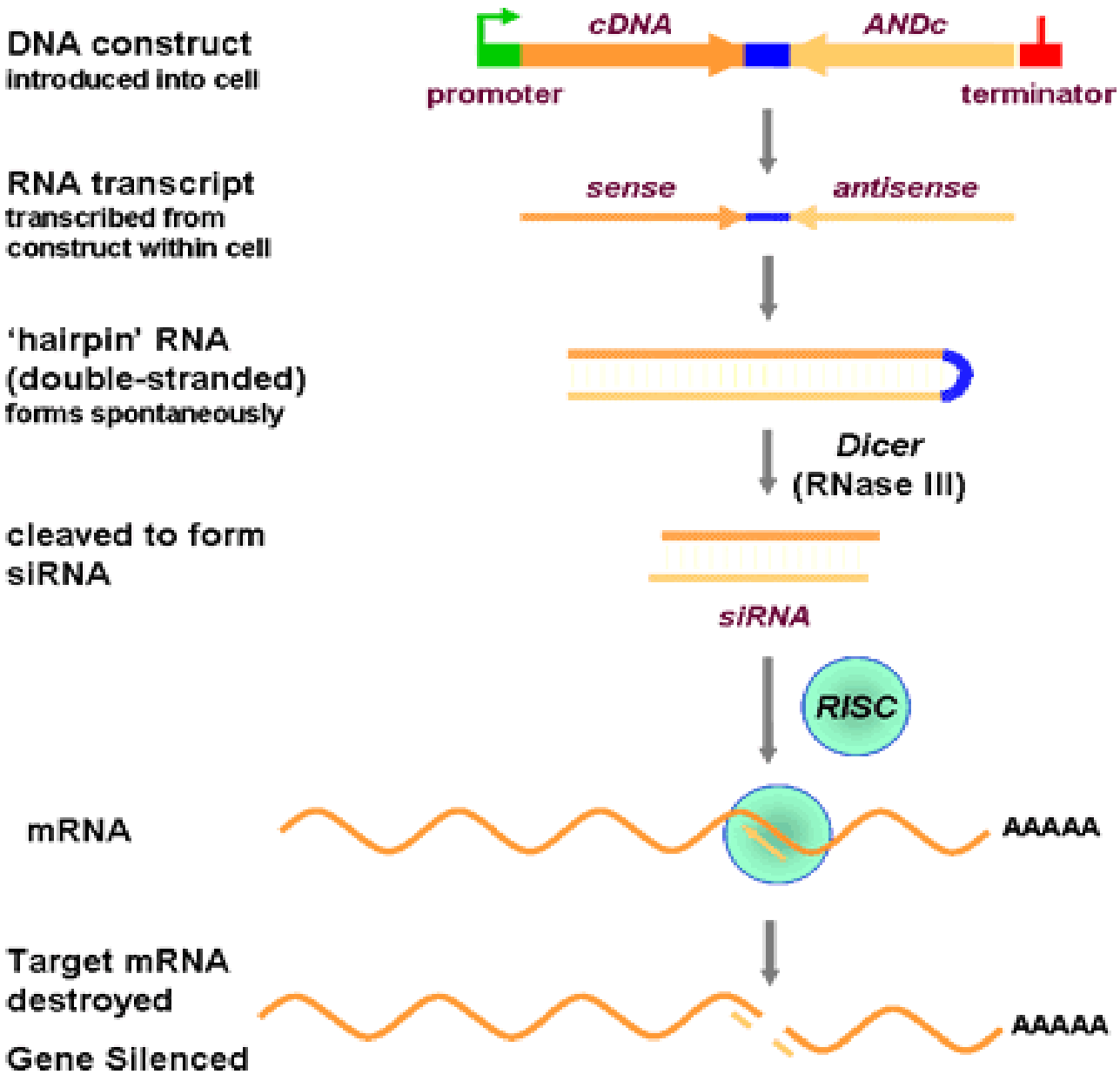
- That effect is known as **gene silencing**, and it persists even in the (first generation) offspring of that affected organism.

## Transgene-dependent Gene Silencing In Plants

- An important aspect of RNA silencing in plants is that it can be triggered locally and then spread via a **mobile silencing signal**

- Systemic spread of silencing also occurs in the organisms; through the mechanism may not be the same as in the plants

- The signaling molecule is not known but is expected to contain a nucleic acid component to account for the sequence specificity.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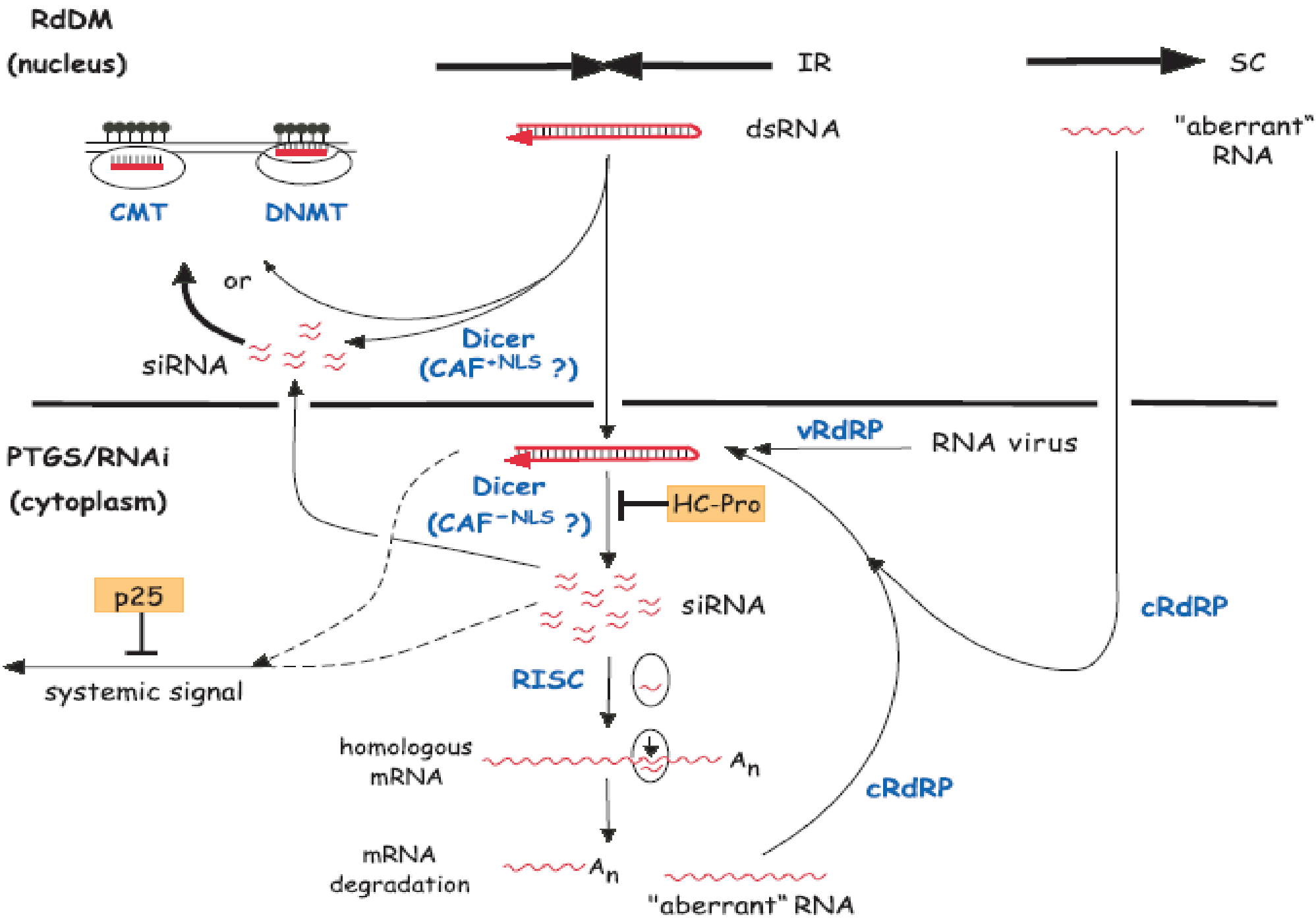
Gene Silenced


RdDM
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systemic signal
RISC
homologous
mRNA
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cRdRP
mRNA
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An
"aberrant" RNA

# RNA Guiding Homologous DNA Modification

- A role for RNA in guiding *de novo* cytosine (C) methylation of homologous DNA sequences was first discovered **in viroid-infected transgenic plants** and subsequently in **nonpathogenic plant systems**

- RNA-directed DNA methylation requires dsRNAs that are cleaved into **small RNAs** similar to those guiding homologous RNA degradation in PTGS/RNAi.

# RNA Guiding Homologous DNA Modification

- Only DNA sequences complementary to the guide RNA become modified, **suggesting** direct **RNA-DNA interactions.**

- DNA sequences as short as 30 base pairs can be targets for methylation, which occurs at all Cs, including those not present in symmetrical CpG (and in plants, CpNpG) nucleotide groups , which are the conventional substrates for methylation.

- Any DNA sequence can apparently become modified by RNA-directed DNA methylation, even ones that are not usually thought to be transcribed, such as promoters.

# RNA Guiding Homologous DNA Modification

- dsRNAs that contain promoter sequences are thus able to direct methylation and transcriptional silencing of homologous promoters in trans
- Moreover, RNAs produced in the cytoplasm as a consequence of PTGS can enter the nucleus and trigger homologous DNA methylation
- In some instances of PTGS, RNA-directed DNA methylation might be required for initiation or maintenance of silencing.

# RNA Guiding Homologous DNA Modification

- The protein machinery involved in RNA directed DNA methylation has not yet been determined, but the minimal enzymatic activities presumably include a ***de novo* DNA methyltransferase** (**DNMT**) and an RNA helicase to unwind dsRNA.

- Whether the dsRNA or the small RNA degradation products are required for RNA-directed DNA methylation is not yet known.

# RNA Guiding Homologous DNA Modification

- Several studies suggest the involvement of small RNAs, which might have ready access to **partially unwound DNA to form an either:**
  - **RNA-DNA duplex** and **single-stranded DNA loop**,
  - **RNA-DNA triple helix**.

- These unusual structures might attract **MTase**.

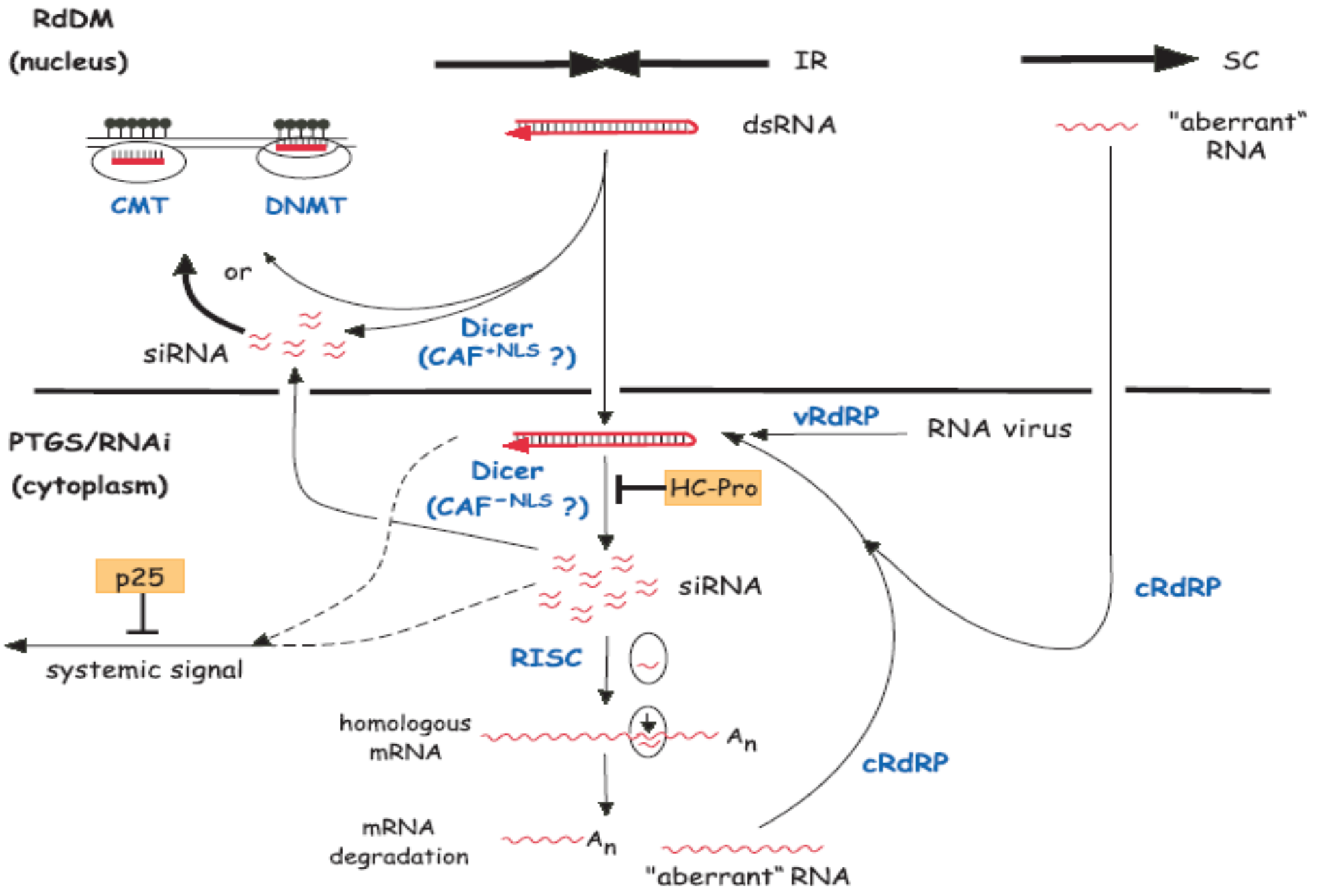
# RNA Guiding Homologous DNA Modification

- Alternatively, small RNAs could interact with MTase and guide the enzyme to a homologous DNA sequence.
- A possible candidate MTase is the so-called [CMT] **chromomethylase**, a special chromodomain-containing plants
- Chromodomains are believed to mediate interactions between chromatin regulatory proteins.
- Intriguingly, the chromodomain MTase that has been found so far only in of the **histone acetylase** MOF in *Drosophila* has been shown to act as an RNA interaction module

# RNA Guiding Homologous DNA Modification

- Conceivably, small RNAs might interact with a chromomethylase through the chromodomain to direct methylation of homologous DNA sequences or to maintain methylation at non-CpGs

- Whether RNA-binding ability is a general property of chromodomains remains to be seen.


RdDM
(nucleus)
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RNA
CMT
DNMT
or
siRNA
Dicer
(CAF+NLS ?)
PTGS/RNAi
(cytoplasm)
vRdRP
RNA virus
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(CAF-NLS ?)
HC-Pro
p25
siRNA
cRdRP
systemic signal
RISC
homologous
mRNA
An
cRdRP
mRNA
degradation
An
"aberrant" RNA

## Cellular protein involved in gene silencing

- Proteins with homology to **RNA-directed RNA polymerases (cRdRP)** function in post-transcriptional gene silencing:
  - in quelling in the fungus *Neurospora crassa*,
  - RNAi in the nematode *Caenorhabditis elegans*, and
  - co-suppression in the mustard plant *Arabidopsis thaliana*.

- These findings are consistent with a conserved mechanism operating in these diverse species.

## Cellular protein involved in gene silencing

- **Aberrant RNAs** are presumed be either improperly spliced or terminated.

- Aberrant RNAs that are mis-spliced and polyadenylated irregularly have been detected in a chalcone synthase PTGS system in petunia

- A new aspect of silencing processes is the possible link with **nonsense-mediated decay** (NMD), an evolutionarily conserved pathway in which mRNAs that contain a premature stop codon are selectively degraded.

## Cellular protein involved in gene silencing

- Antisense constructs probably also produce RNAs that feed into the dsRNA-induced degradation pathway.

- **A DEAH-box RNA helicase** (Mut*6*) that is involved in degrading misspliced and non-polyadenylated transcript was shown to be required for transgene and transposon silencing in the unicellular green alga *Chlamydomonas reinhardtii*

# Cellular protein involved in gene silencing

- These results suggest a partial overlap between nonsense-mediated decay (**NMD**) and **PTGS** pathways and provide new insights for unraveling the PTGS pathway.

- Cellular proteins involved in PTGS are being widely studied and will be one of the exciting prospects for the future in plant molecular genetics.

# Cellular proteins involved in posttranscriptional gene silencing

| Protein | Mutant | Species | References |
|---|---|---|---|
| RdRp | *qde1* | *Neurospora* | Cogoni et al 1999 |
| ‘‘ | *sde1* | *Arabidopsis* | Dalmay et al 2000 |
| ‘‘ | *sgs2* | *Arabidopsis* | Mourrain et al 2000 |
| ‘‘ | *ego1* | *C. elegans* | Smardon et al 2000 |
| eIF2C-like | *qde2* | *Neurospora* | Catalanotto et al 2000 |
| ‘‘ | *Rde1* | *C. elegans* | Tabara et al 1999 |
| ‘‘ | *ago1* | *Arabidopsis* | Fagard et al 2000 |
| RecQ DNA | *qde3* | *Neurospora* | Cogoni et al 1999 |
| RNase D-like | *mut-7* | *C. elegans* | Ketting et al 1999 |
| RNA helicase | *mut-6* | *Chlamydomonas* | Wu-Scharf et al 2000 |
| Coiled-coil Protein | *Sgs3* | *Arabidopsis* | Mourrain et al 2000 |
| NMD Proteins | *smg2,5,6* | *C. elegans* | Domeier et al 2000 |
| rgs-CaM | No | *Nicotiana tabacum* | Domeier et al 2000 |

***ago1*** argonaute1; ***ego1***, enhancer of glp-1; ***qde***, quelling defective; ***rde***, RNA interference deficient; **rgs-CaM,** regulator of gene silencing calmodulin-like protein; **sde**, silencing defective; ***sgs***, suppressor of gene silencing

# Viral suppressors of posttranscriptional gene silencing

- The potential for using **viral suppressors of PTGS** to piece together silencing pathways and to identify cellular components is just beginning to be realized.

- The natural role of PTGS as an antiviral defense had been reported because **many plant viruses encoding proteins suppress gene silencing**

- Different viral suppressors act at distinct steps in PTGS and can help to elucidate the silencing pathway.

# Viral suppressors of posttranscriptional gene silencing

- The viral suppressors such as, **the helper component protease** (**HC-Pro) of potyviruses** and **the p25 cell movement protein of potato virus X (PVX),** have been particularly informative about the underlying mechanisms of PTGS.

- Investigations on virus suppressors showed that a **mobile silencing signal** is produced in PVX-induced PTGS.
  - However, the systemic silencing induced by PVX could not be detected unless the coding region of p25 was either deleted or modified
  - These results suggest that the **PVX p25 protein blocks PTGS** by **suppressing the cellular RdRP** branch of the pathway

- A cytoplasmic RNA virus vector carrying 35S promoter sequences was able to induce methylation and TGS of nuclear transgenes under the control of the 35S promoter in *Nicotiana benthamiana*.

# Viral suppressors of posttranscriptional gene silencing

- On the basis of the patterns of suppression produced by viral suppressors, some of them affect silencing differently from either HC-Pro or PVX p25 and are likely to define additional steps in the PTGS pathway.

- The obligatory nuclear localization of the **cucumber mosaic virus 2b protein** should help to identify steps of PTGS that occur in the nucleus.

- The host defense function of RNA-mediated silencing is demonstrated by the increased of sensitivity of *Arabidopsis* PTGS mutants to some viruses and the mobilization of transposons in the *Mut6* mutant *Chlamydomonas*

# Viral suppressors of posttranscriptional gene silencing

- Apart from an enhanced susceptibility to viral infection, *Arabidopsis sgs/sde* mutants appear normal.

- However, expression of HC-Pro or over-expression of rgs-CaM causes developmental aberrations in *Nicotiana* species and *Arabidopsis ago1* mutants exhibit marked developmental abnormalities and are infertile

- Although the PTGS pathway appears to be as a whole dispensable for development, the phenotypic irregularities found in a subset of cases where silencing is blocked; **suggest** that PTGS and development share common enzymes or pathways.

# Viral suppressors of posttranscriptional gene silencing

- Determining the extent to which PTGS and RdDM contribute to normal plant development, and not just host defense, is one of the most exciting prospects for the future.
- The powerful tools provided by viral suppressors of silencing and the steadily growing collection of silencing-defective mutants promise a continuation of the rapid progress that has become the norm in plant gene silencing research.
- A prominent feature of PTGS suppression by **HC-Pro** is the absence of the small RNAs associated with silencing
- Grafting experiments have shown that HC-Pro suppression of PTGS does not interfere with either the production or movement of the silencing signal but prevents the plant from responding to that signal

# Viral suppressors of posttranscriptional gene silencing

- HC-Pro suppression of PTGS occurs downstream of the mobile silencing signal at a step preceding the accumulation of the small RNAs

- A study using the yeast two-hybrid system has identified a plant **calmodulin-related protein** (rgs-CaM) that interacts with HC-Pro

- This calmodulin-related protein suppresses gene silencing and might be a cellular intermediary of HC-Pro suppression of PTGS

# Viral suppressors of posttranscriptional gene silencing

- Because calmodulin and related proteins normally act by binding calcium and subsequently activating target proteins, HC-Pro suppression of PTGS possibly occurs via activation of rgs-CaM and its unknown target protein

- In contrast to **HC-Pro, the PVX p25 protein appears to suppress PTGS by targeting the mobile silencing signal**.

- The small sense and antisense RNAs associated with silencing derive from cleavage of dsRNA and a specific

# Viral suppressors of posttranscriptional gene silencing

- Small RNAs accumulate during both virus and transgene-induced gene silencing:
  - **HC- Pro** suppression of silencing interferes with accumulation of the small RNAs but ***does not eliminate*** either the production or movement of the silencing signal
  - **PVX p25 protein** interferes with the mobile silencing signal, but does ***not affect the accumulation of small RNAs*** produced in the viral RdRP dependent branch of PTGS.

- The obligatory nuclear localization of the cucumber mosaic virus 2b protein should help to identify steps of PTGS that occur in the nucleus.

# Viral suppressors proteins

- Several other **viral proteins** have now been identified as inhibitors of the PTGS:
  1. The AC2 protein encoded in the ***African cassava mosaic virus*** (ACMV)
  2. The 19-KDa protein of ***Tomato bushy stunt virus*** (TBSV)
  3. The P1 protein of the ***Rice yellow mottle virus*** (RYMV)

# RNA interference and **Gene Silencing**

- **RNAi** is one methodology which can be utilized by scientists to cause **gene silencing** / **knockout**.

- **knockdown** ??!!!! slowing

- During 2002, Thomas A. Rosenquist and Gregory J. Hannon created "**knockdown**" mice via genetic engineering so those mice (continually) produced the dsRNA which silenced a selected gene.

# RNA interference

- Later, the first-generation offspring of those **knockdown mice** also "silenced" that selected gene in their bodies.

- **Knockdown** refers to (a scientist's) alteration of a particular gene within an organism, so that specific gene may subsequently **not be expressed**, or be **expressed only under (controlled) condition(s) selected by that scientist.**

# RNA interference

- **RNA interference (RNAi)** is a recently identified mechanism that influences expression of genes in a wide variety of organisms, including fungi, nematodes, fruit fly (Drosophila), plants and mammalian cells.
- RNAi has been implicated in several, different processes including:
  1. the temporal regulation of developmental gene expression,
  2. the protection of genome integrity by prevention of transposon mobilization.
  3. In addition, in plants, RNAi also functions as a resistance mechanism against virus infection by targeting virus-specific RNA molecules for degradation.

# RNA Interference and Gene Silencing: History and Overview

- **Post-transcriptional gene silencing (PTGS),** which was initially considered a bizarre phenomenon limited to petunias and a few other plant species, is now one of the hottest topics in molecular biology
- In the last few years, it has become clear that PTGS occurs in both plants and animals and has roles in viral defense and transposon silencing mechanisms.
- Perhaps most exciting, however, is the emerging use of PTGS and, in particular, RNA interference (RNAi) — PTGS initiated by the introduction of double-stranded RNA (dsRNA) — as a tool to knock out expression of specific genes in a variety of organisms

# RNA Interference and Gene Silencing: History and Overview

- The first evidence that dsRNA could lead to gene silencing came from work in the nematode *Caenorhabditis elegans*.
- Seven years ago, researchers Guo and Kemphues were attempting to use antisense RNA to shut down expression of the par-1 gene in order to assess its function.
- As expected, injection of the antisense RNA disrupted expression of par-1, but quizzically, injection of the sense-strand control did too

# RNA Interference and Gene Silencing: History and Overview

- It was then that Fire and Mello first injected dsRNA — a mixture of both sense and antisense strands — into *C. elegans* resulted in much **more efficient silencing** than injection of either the sense or the antisense strands alone.
- Injection of just a **few molecules of dsRNA per cell** was sufficient to completely silence the homologous gene's expression.
- Furthermore, injection of dsRNA into the gut of the worm caused gene silencing not only throughout the worm, but also in its first generation offspring .

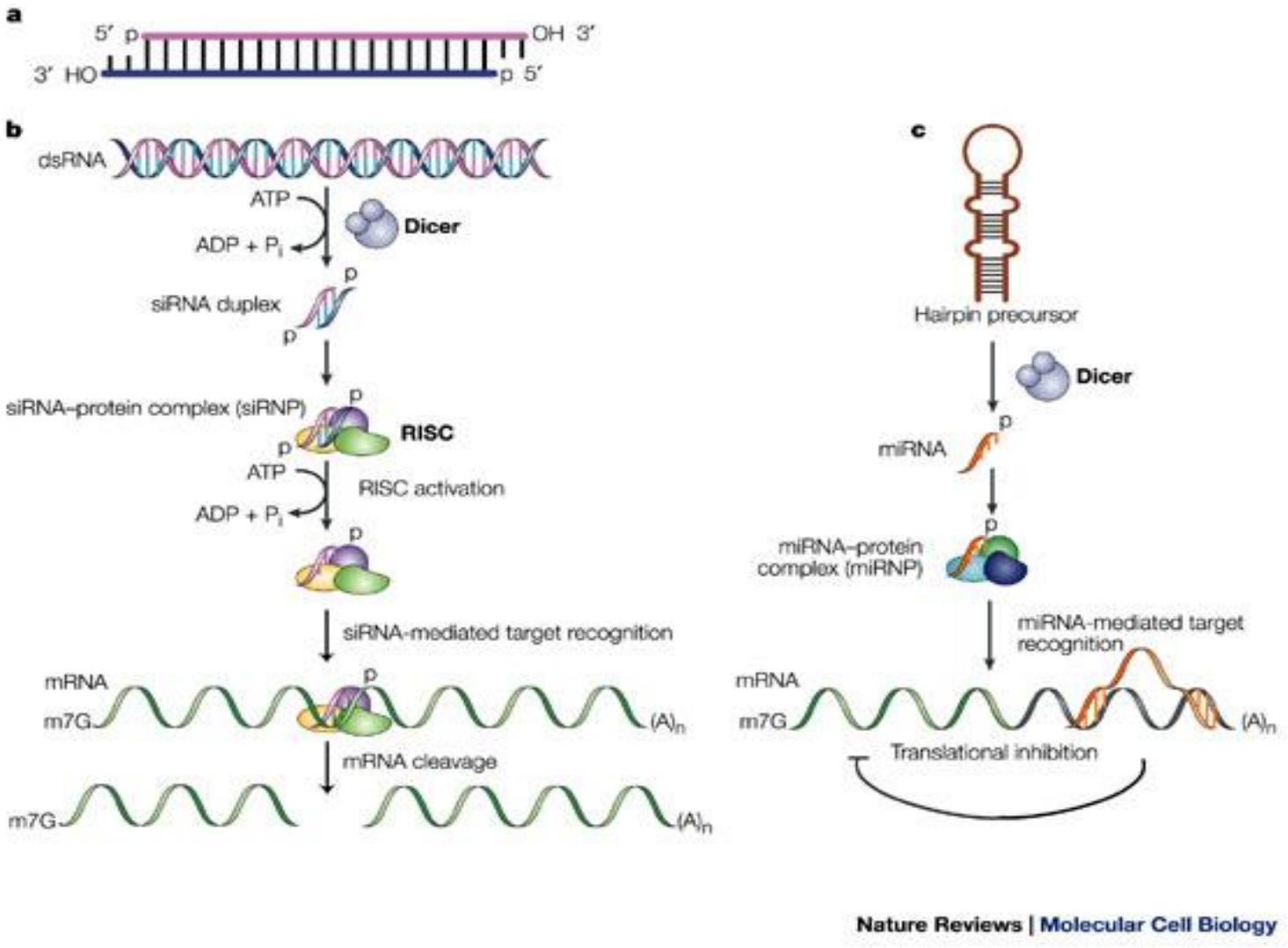
# RNA Interference and Gene Silencing: History and Overview

- The potency of RNAi inspired Fire and Timmons to try feeding nematodes bacteria that had been engineered to express dsRNA homologous to the *C. elegans* unc-22 gene.
  - Surprisingly, these worms developed an unc-22 null-like phenotype
- Further work showed that soaking worms in dsRNA was also able to induce silencing .
- These strategies, whereby large numbers of nematodes are exposed to dsRNA, have enabled large-scale screens to select for RNAi-defective *C. elegans* mutants and have led to large numbers of gene knockout studies within this organism

# RNA Interference and Gene Silencing: History and Overview

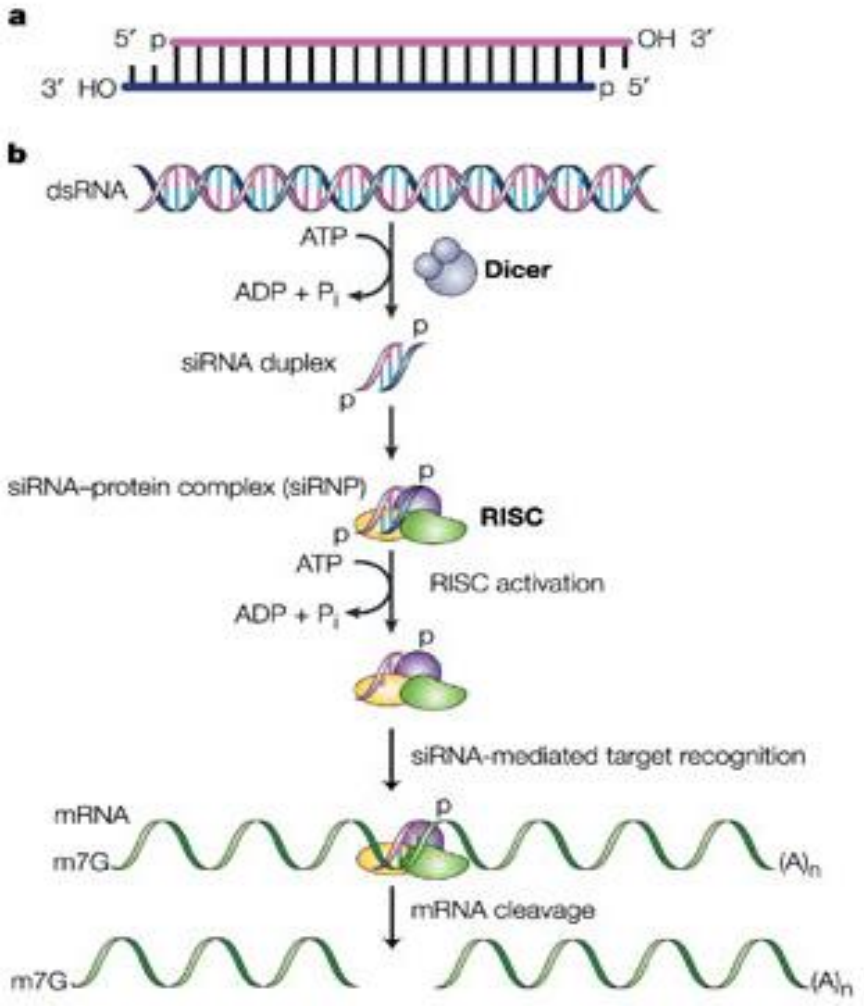
- RNAi has also been observed in Drosophila.
  - Although a strategy in which yeast were engineered to produce dsRNA and then fed to fruit flies failed to work, microinjecting Drosophila embryos with dsRNA does effect silencing
- Silencing can also be induced by "shooting" dsRNA into Drosophila embryos with a "gene gun" or by engineering flies to carry DNA containing an inverted repeat of the gene to be silenced.

# Current Models of the RNAi Mechanism



# Current Models of the RNAi Mechanism

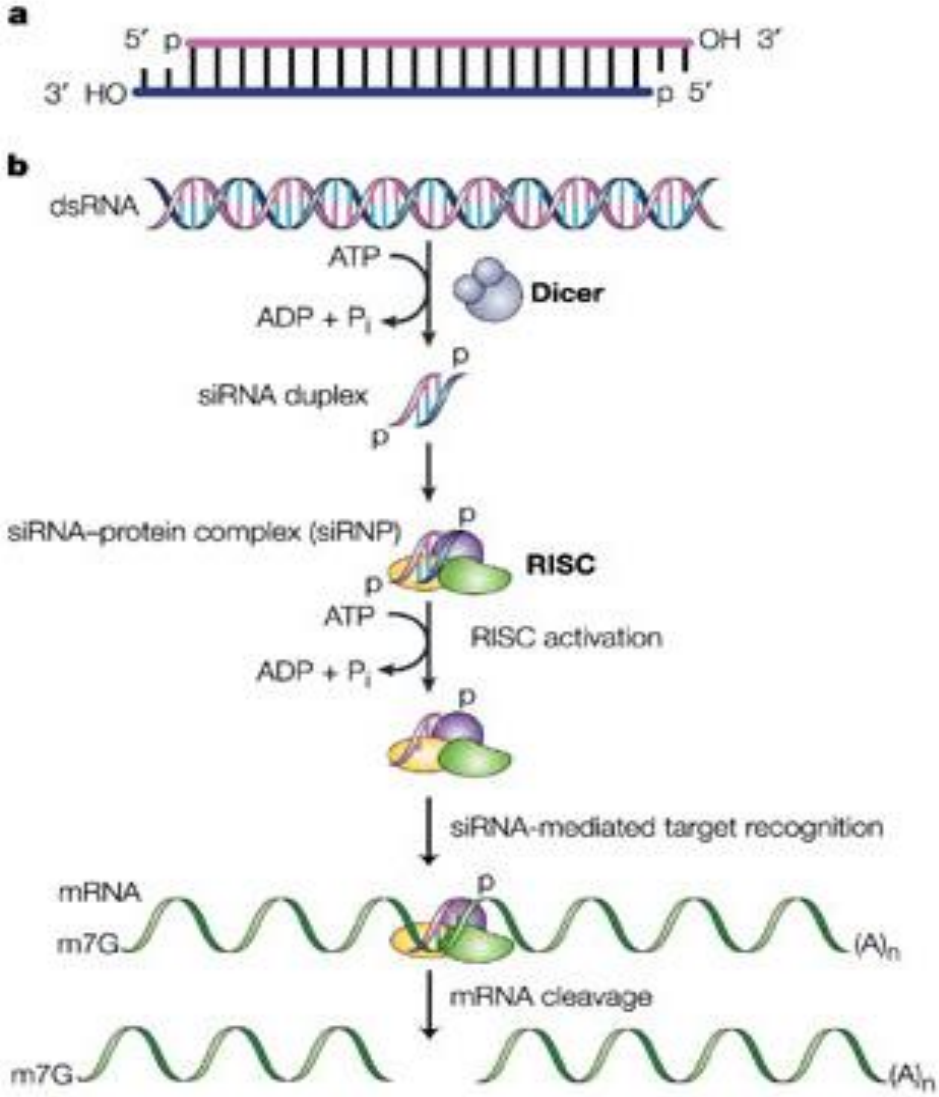
- the current models of the RNAi mechanism, RNAi includes both **initiation** and **effector** steps.
- In the initiation step, input dsRNA is digested into 21-23 nucleotide small interfering RNAs (siRNAs), which have also been called "guide RNAs"
- Evidence indicates that siRNAs are produced when the enzyme Dicer cleaves dsRNA in an ATP-dependent, processive manner.
- Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNAs), each with 2-



# Current Models of the RNAi Mechanism

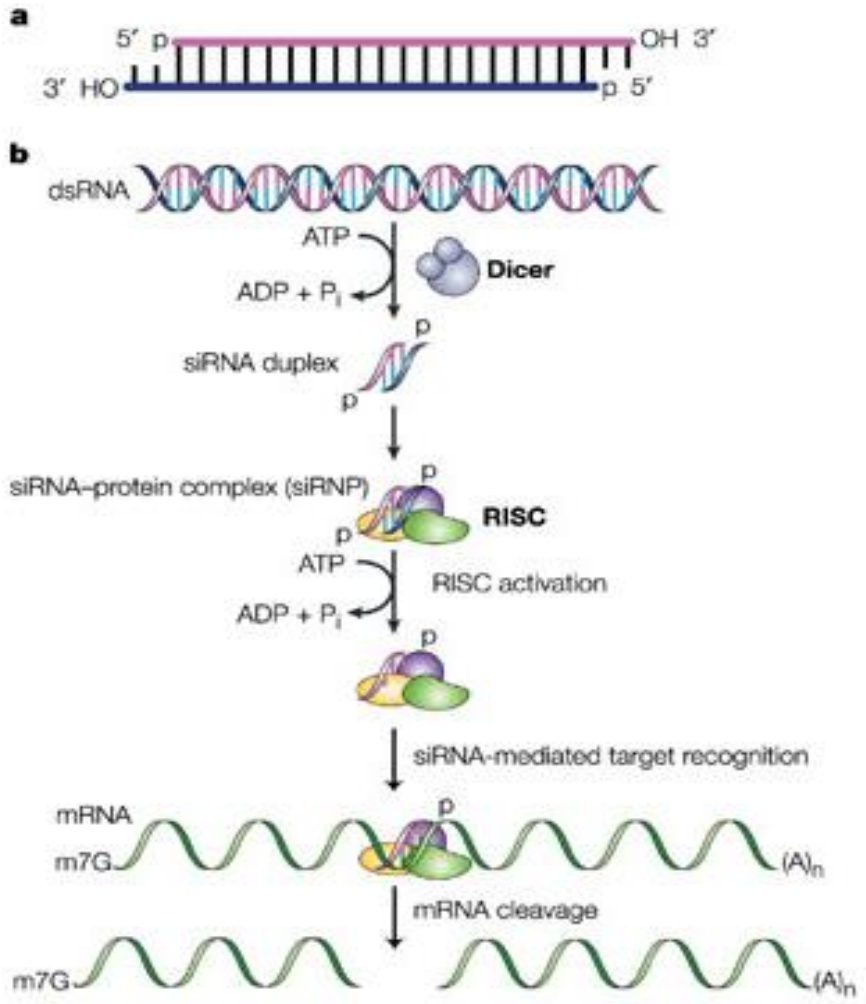
- In the effector step, the siRNA duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, or **RISC**.

- An ATP-depending unwinding of the siRNA duplex is required for activation of the RISC.



# Current Models of the RNAi Mechanism

- The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA
- Although the mechanism of cleavage is at this date **unclear**, research indicates that each RISC contains a single siRNA and an **RNase** that appears to be distinct from Dicer



# Current Models of the RNAi Mechanism

- Because of the remarkable potency of RNAi in some organisms, an amplification step within the RNAi pathway has also been proposed.
- Amplification could occur by either :
  - copying of the input dsRNAs, which would generate more siRNAs,
  - replication of the siRNAs themselves.

# The Genes and Enzymes Involved in PTGS and RNAi

- Possible Role for cellular RNA-dependent RNA Polymerase: Several of these, including Neurospora qde-1, Arabidopsis SDE-1/SGS-2 and C. elegans ego-1, appear to encode RNA-dependent RNA polymerases (RdRPs).
- RNAi Initiators
  - Two *C. elegans* genes**, rde-1** and **rde-4** , are believed to be involved in the initiation step of RNAi.
- RNAi Effectors
  - Important genes for the effector step of PTGS include the C. elegans rde-2 and mut-7 genes. These genes were initially identified from heterozygous mutant worms that were unable to transmit RNAi to their homozygous offspring

***ago1*** argonaute1; ***ego1***, enhancer of glp-1; ***qde***, quelling defective; ***rde***, RNA interference deficient; **rgs-CaM,** regulator of gene silencing calmodulin-like protein; **sde**, silencing defective; ***sgs***, suppressor of gene silencing

# Cellular proteins involved in posttranscriptional gene silencing

| Protein | Mutant | Species | References |
|---|---|---|---|
| RdRp | *qde1* | *Neurospora* | Cogoni et al 1999 |
| ‘‘ | *sde1* | *Arabidopsis* | Dalmay et al 2000 |
| ‘‘ | *sgs2* | *Arabidopsis* | Mourrain et al 2000 |
| ‘‘ | *ego1* | *C. elegans* | Smardon et al 2000 |
| eIF2C-like | *qde2* | *Neurospora* | Catalanotto et al 2000 |
| ‘‘ | *Rde1* | *C. elegans* | Tabara et al 1999 |
| ‘‘ | *ago1* | *Arabidopsis* | Fagard et al 2000 |
| RecQ DNA | *qde3* | *Neurospora* | Cogoni et al 1999 |
| RNase D-like | *mut-7* | *C. elegans* | Ketting et al 1999 |
| RNA helicase | *mut-6* | *Chlamydomonas* | Wu-Scharf et al 2000 |
| Coiled-coil Protein | *Sgs3* | *Arabidopsis* | Mourrain et al 2000 |
| NMD Proteins | *smg2,5,6* | *C. elegans* | Domeier et al 2000 |
| rgs-CaM | No | *Nicotiana tabacum* | Domeier et al 2000 |

***ago1*** argonaute1; ***ego1***, enhancer of glp-1; ***qde***, quelling defective; ***rde***, RNA interference deficient; **rgs-CaM,** regulator of gene silencing calmodulin-like protein; **sde**, silencing defective; ***sgs***, suppressor of gene silencing

# Non-specific Gene Silencing by Long dsRNAs

- While the natural presence of RNAi had been observed in a variety of organisms (plants, protozoa, insects, and nematodes), evidence for the existence of RNAi in mammalian cells took longer to establish.
  - Transfection of long dsRNA molecules (>30 nt) into most mammalian cells **causes nonspecific suppression of gene expression**, as opposed to the gene-specific suppression seen in other organisms.
- This suppression has been attributed to an **antiviral response**, which takes place through one of two pathways.
  - In one pathway, long dsRNAs activate a protein kinase, PKR.
    - Activated PKR, in turn phoshorylates and inactivates the translation initiation factor, eIF2a, leading to repression of translation.

# RNA interference – why?

- Plant Biotechnology
- Studying gene function
  - Knockout or inhibit a gene's normal function
  - What phenotypic changes are observed?
- Therapeutic suppression
  - cancer treatment

# How do scientists benefit from RNAi in fighting cancer?

- Current clinical trials of scientists are targeted to use siRNA against viruses (i.e. adenoviruses) that code for protein which bind to P53 protein and inactivate it.
- Others aim to silence Her2 gene that play an important role in the oncogenesis of several types of cancer. Researchers designed three siRNA to act against Her2 gene.
- In vitro studies showed that introduction of siRNA greatly reduced cell surface expression of Her2 protein.

# Glossary of Terms

- **Cosuppression -** Silencing of an endogenous gene caused by the introduction of a transgene or infection by a virus.
  - This term, which can refer to silencing at the post-transcriptional (PTGS) or transcriptional (TGS) level, has been primarily adopted by researchers working with plants.

- **Post-transcriptional Gene Silencing (PTGS) -** Silencing of an endogenous gene caused by the introduction of a homologous dsRNA, transgene or virus.
  - In PTGS, the transcript of the silenced gene is synthesized but does not accumulate because it is rapidly degraded.
  - This is a more general term than RNAi, since it can be triggered by several different means.

# Glossary of Terms

- **Quelling -** PTGS in *Neurospora crassa* induced by the introduction of a transgene.

- **RISC -** RNA-induced silencing complex. A nuclease complex, composed of proteins and siRNA, that targets and destroys endogenous mRNAs complementary to the siRNA within the complex.

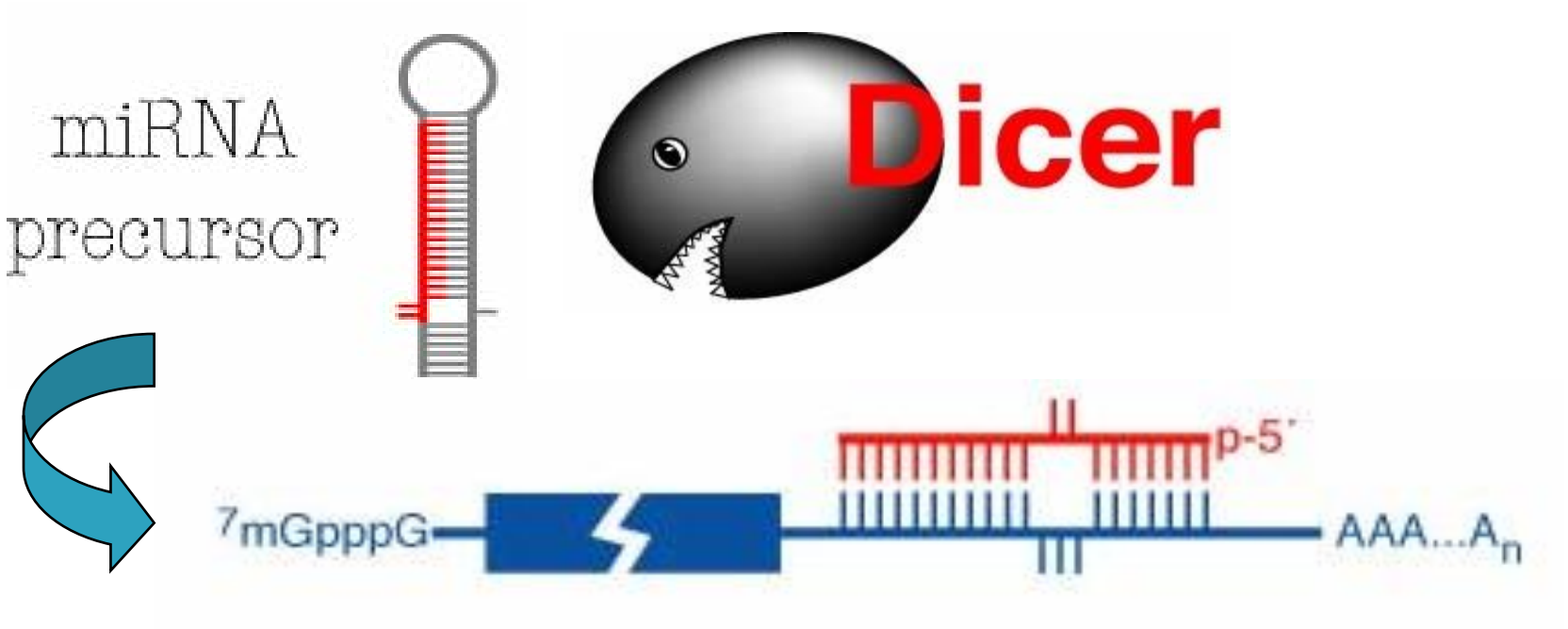
- **RNA interference (RNAi) -** Post-transcriptional gene silencing (PTGS) induced by the direct introduction of dsRNA.
  - The term "RNA interference" was first used by researchers studying *C. elegans.*

# Glossary of Terms

- **siRNAs - Small interfering RNAs**.
  - Current models of PTGS indicate that these 21-23 nucleotide dsRNAs mediate PTGS.
  - Introduction of siRNAs can induce PTGS in mammalian cells.
  - siRNAs are apparently produced in vivo by cleavage of dsRNA introduced directly or via a transgene or virus.
  - Amplification by an RNA-dependent RNA polymerase (RdRP) may occur in some organisms.
  - siRNAs are incorporated into the RNA-induced silencing complex (RISC), guiding the complex to the homologous endogenous mRNA where the complex cleaves the transcript.

# micro RNA (miRNA)

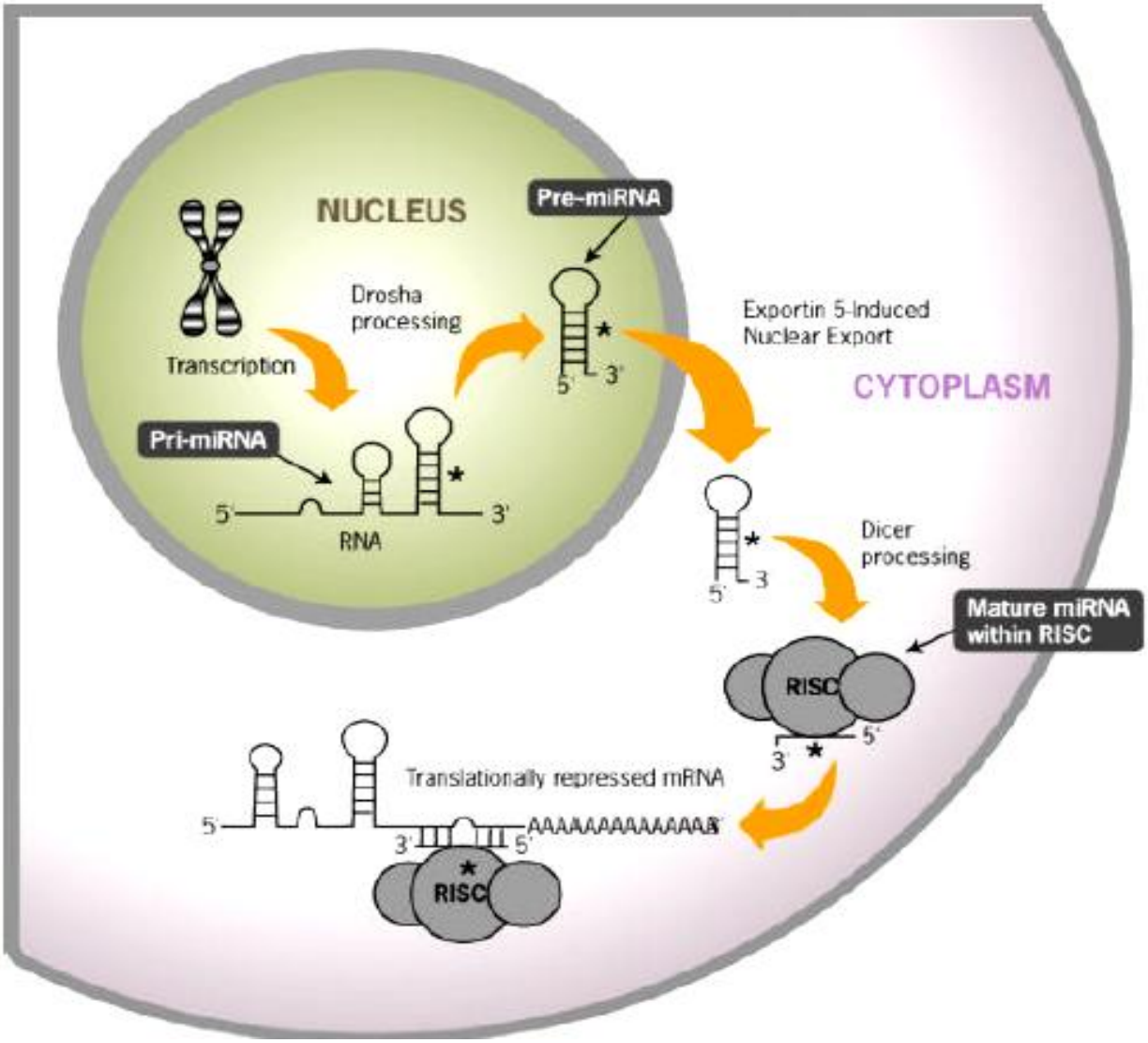
- Gene expression regulation
- Created by similar process to siRNA
- Generally prevents binding of ribosome



# MicroRNAs (miRNAs)

- Recently, nearly 100 discovered ~22 nt RNA molecules, termed microRNAs (miRNAs), were identified in Drosophila, C. elegans, and HeLa cells
- Much like lin-4 and let-7, these miRNAs are formed from precursor RNA molecules that fold into a stem-loop secondary structure.
- The newly discovered ~22 nt miRNAs are believed to play a role in regulation of gene expression, and at least two of them are known to require Dicer for their production
- It appears that the use of small RNAs for both gene regulation and RNAi is a common theme throughout evolution.

# miRNA Expression

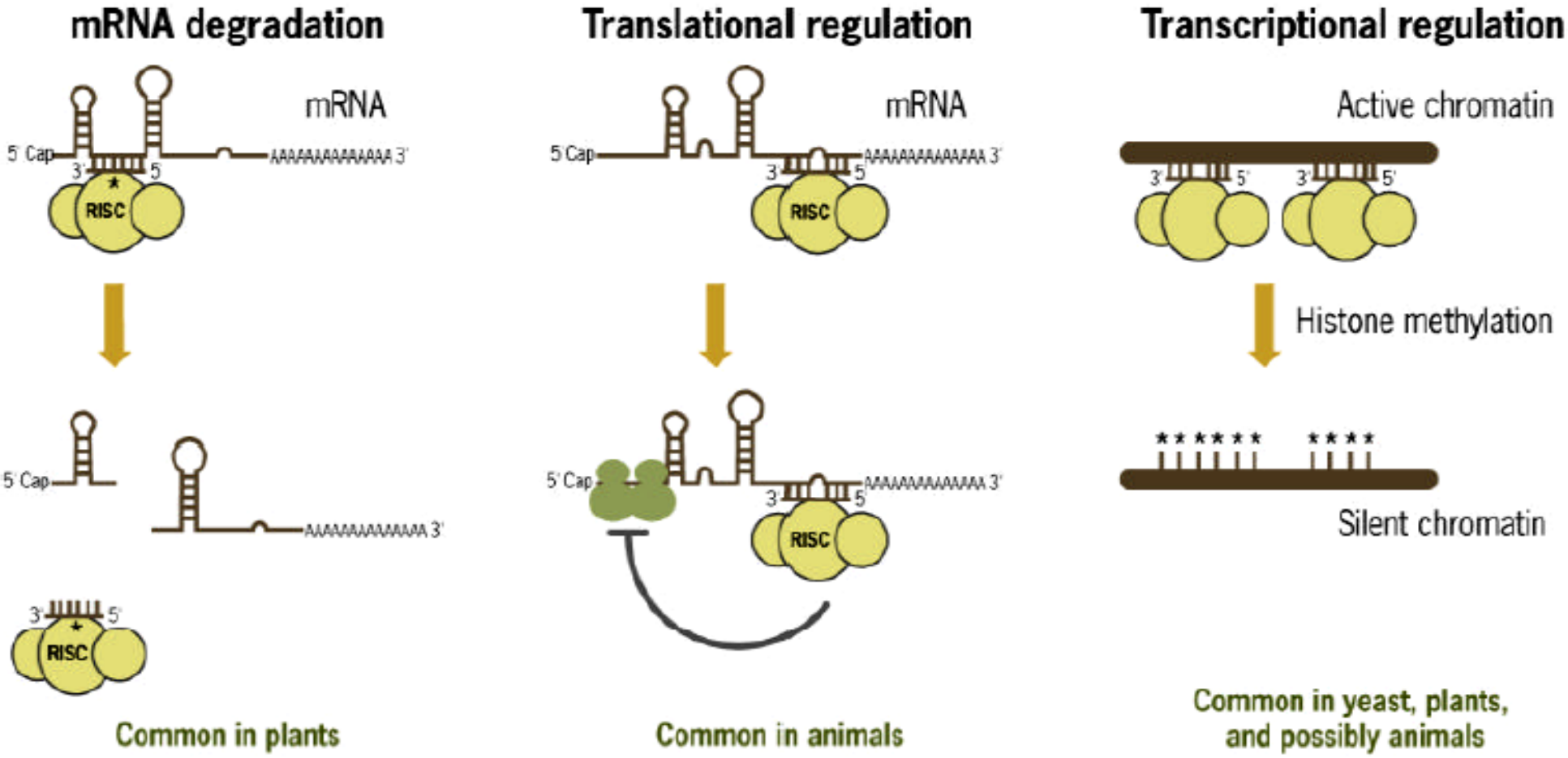


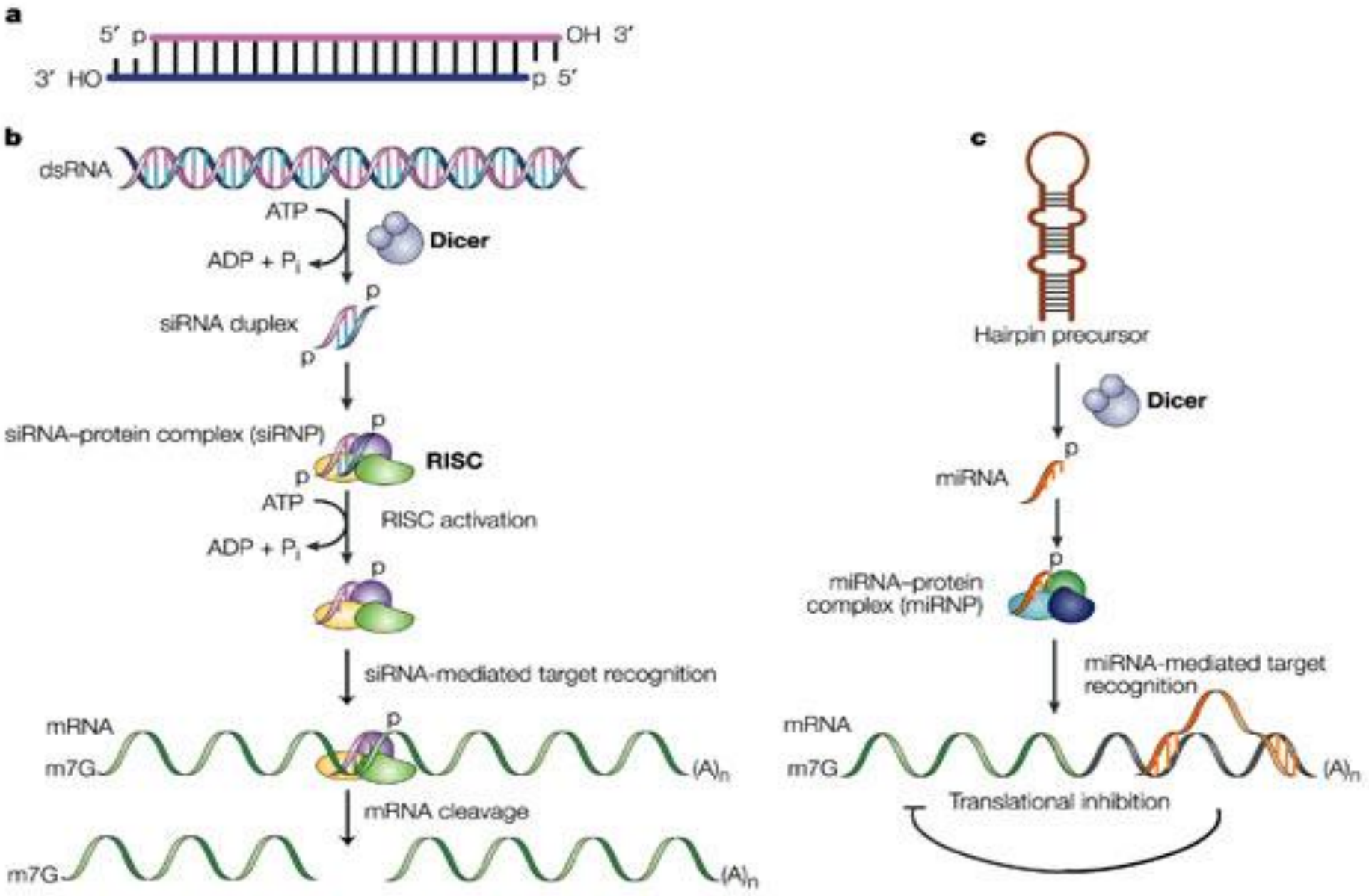

Mammalian genomes are estimated to encode as many as 1000 unique miRNAs

miRNAs are predicted to Directly regulate the expression of at least 30% of all human protein -encoding genes (Lewis 2005*)

*Cell. 2005 Jan 14;120(1):15-20

# Mechanisms of Small RNA-Induced Gene Regulation

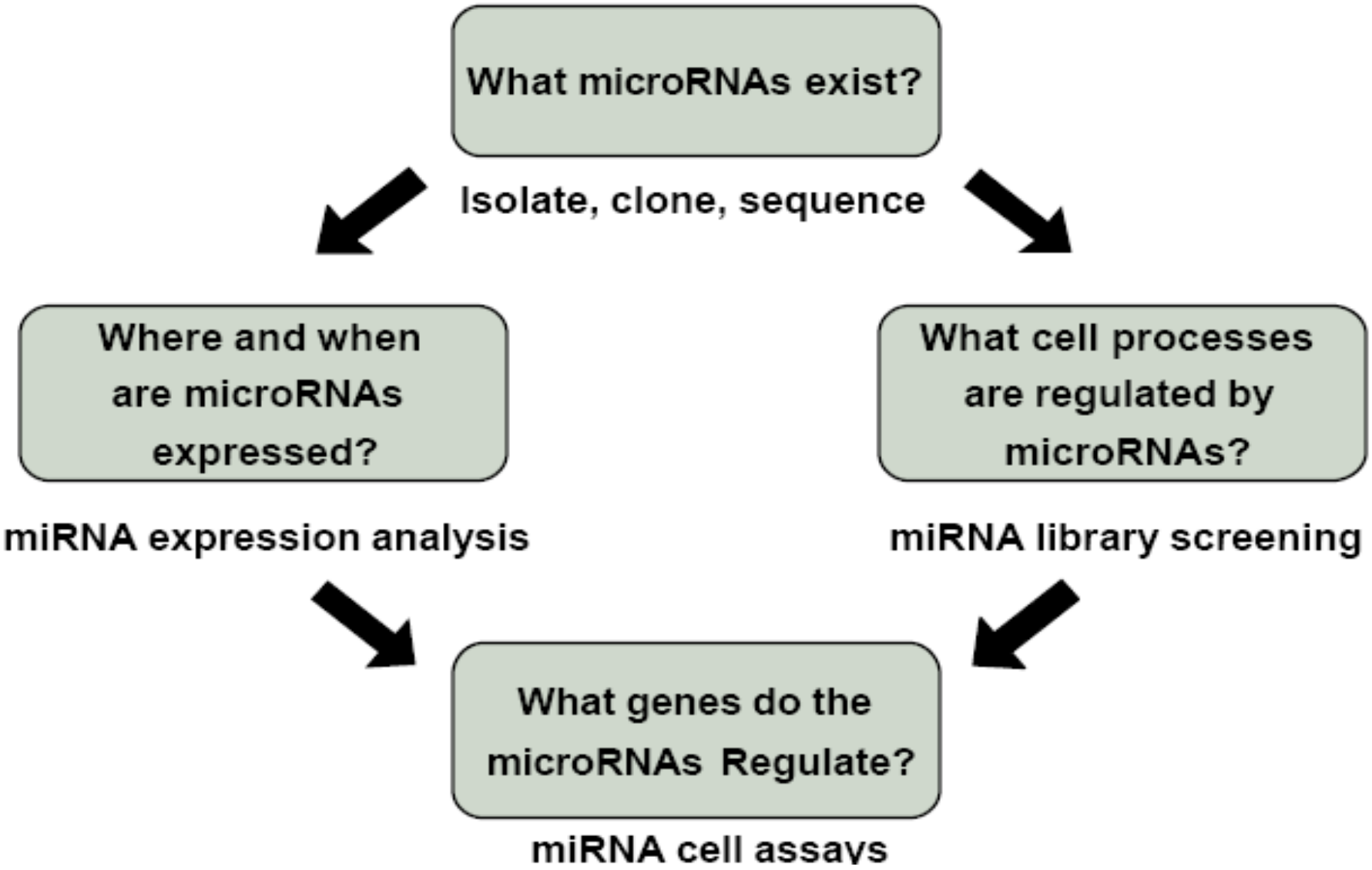



a
5′ p
OH 3′
3′ HO
p 5′
b
dsRNA
ATP
ADP + Pi
Dicer
siRNA duplex
siRNA–protein complex (siRNP)
RISC
ATP
ADP + Pi
RISC activation
siRNA-mediated target recognition
mRNA
m7G
(A)n
mRNA cleavage
m7G
(A)n
c
Hairpin precursor
Dicer
miRNA
miRNA–protein complex (miRNP)
miRNA-mediated target recognition
mRNA
m7G
(A)n
Translational inhibition

# Examples of miRNA Functions

| microRNA | Organism | Function |
|---|---|---|
| lin-4 | worm | Early larval development transitions |
| let-7 | worm | Late larval development transitions |
| lsy-6 | worm | Left/right asymmetry of chemoreceptor expression |
| bantam | fly | Growth control during development |
| miR-14 | fly | Apoptosis and fat metabolism |
| miR165/166 | plant | Axial leaf development |
| miR172 | plant | Flower development |
| miR-JAW | plant | Leaf development, embryonic patterning |
| miR159 | plant | Leaf development |
| miR-181 | mouse | Hematopoietic differentiation |

# MicroRNA Research Questions



# miRNA Expression Profiling