Molecular Biology (22ZOOC23)

Structure of DNA

DISCOVERY :

Nucleic acids were discovered in 1869 by the Swiss biochemist Johann Friedrich Miescher (1844–1895). Miescher discovered the presence of an unusual organic compound in the nuclei of cells and gave that compound the name nuclein. The compound was unusual because it contained both nitrogen and phosphorus, in addition to carbon, hydrogen, and oxygen. Nuclein was one of the first organic compounds to have been discovered that contained this combination of elements. Although later research showed that various forms of nuclein occurred in other parts of the cell, the name remained in the modified form by which it is known today: nucleic acid.

More About Miescher's Experiment

- The young Swiss physician Friedrich Miescher just his aim being to elucidate the building blocks of life.
- Choosing leucocytes as his source material, he first investigated the proteins in these cells. However, during these experiments, he noticed a substance with unexpected properties that did not match those of proteins.

Contd..

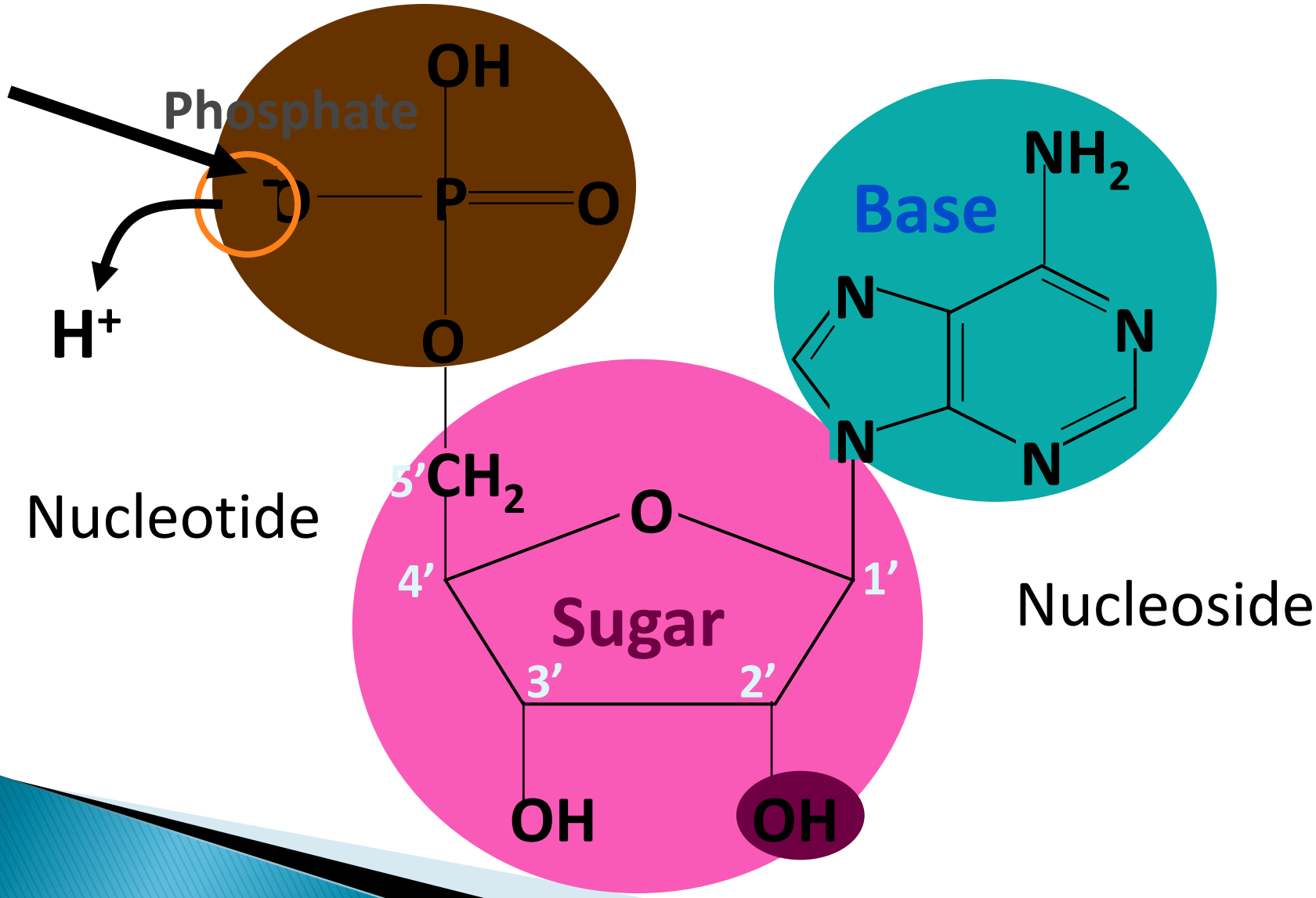
- Miescher had obtained the first crude purification of DNA. He further examined the properties and composition of this enigmatic substance and showed that it fundamentally differed from proteins.
- Due to its occurrence in the cells' nuclei, he termed the novel substance "nuclein"--a term still preserved in today's name deoxyribonucleic acid

Structure of nucleic acids

- Nucleic acids are polymers, very large molecules that consist of much smaller units repeated many times over and over again. The small units of which polymers are made are known as monomers. In the case of nucleic acid, the monomers are called nucleotides.

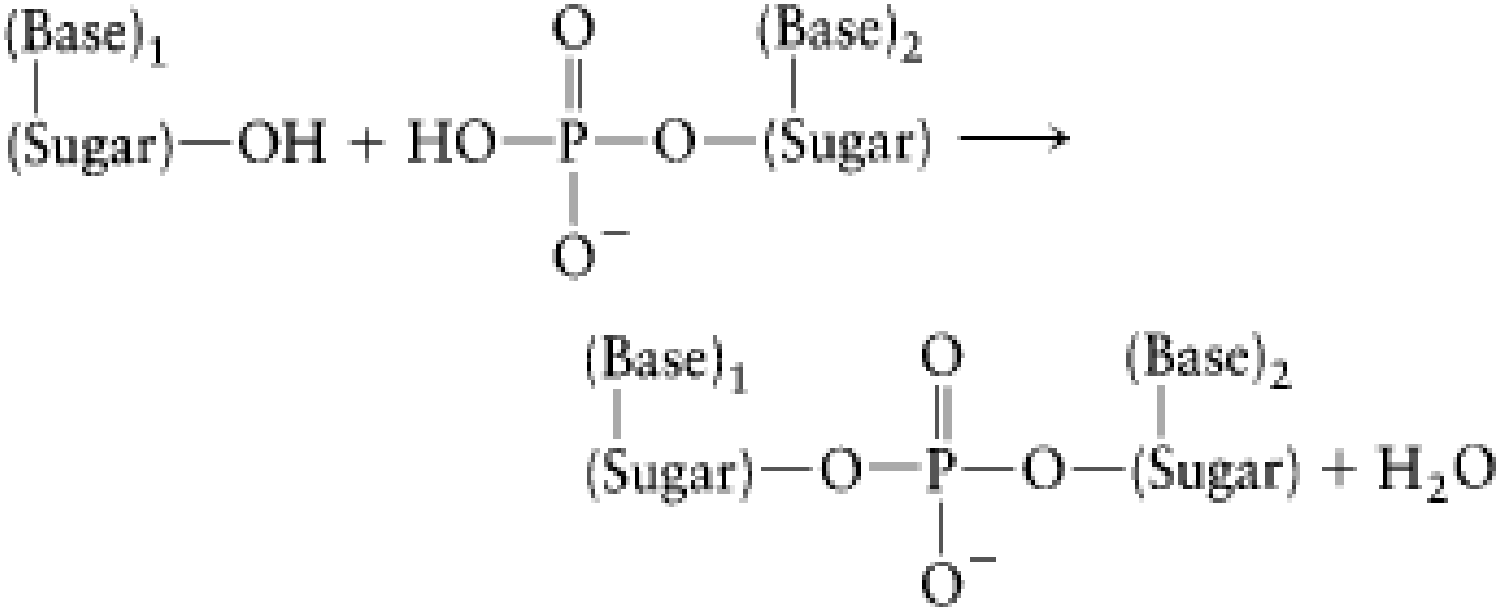
A Nucleotide

Adenosine Mono Phosphate (AMP)



contd..

- When nucleotides polymerize to form nucleic acids, the hydroxyl group attached to the 3′ carbon of a sugar of one nucleotide forms an ester bond to the phosphate of another nucleotide, eliminating a molecule of water:



KINDS OF NUCLEIC ACIDS

- Two different kinds of sugars are found in nucleic acids.
- One kind of sugar is called

  (a) deoxyribose.

  (b) ribose.

The difference between the two compounds is that deoxyribose contains one oxygen less (deoxy means "without oxygen") than does ribose.

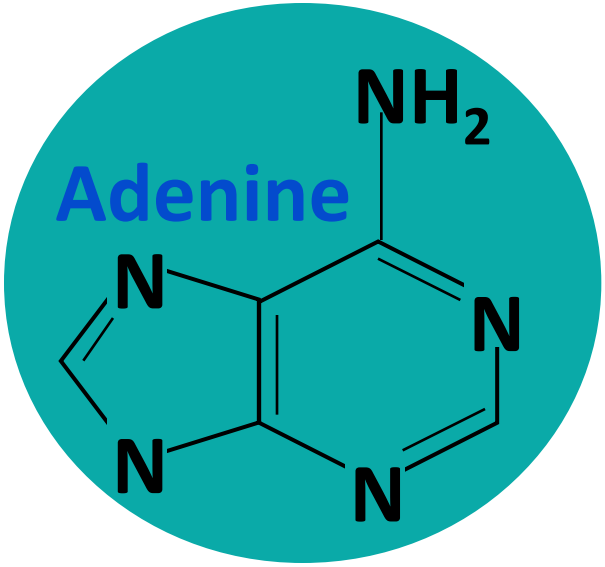
Contd..

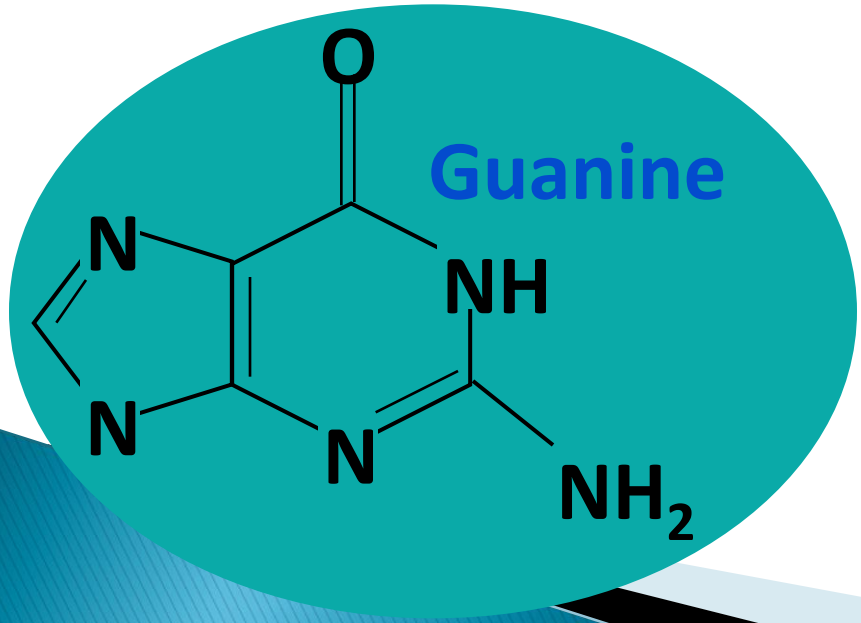
- Nucleic acids that contain the sugar deoxyribose are called deoxyribonucleic acid, (DNA)
- Those that contain ribose are called ribonucleic acid (RNA)

DNA

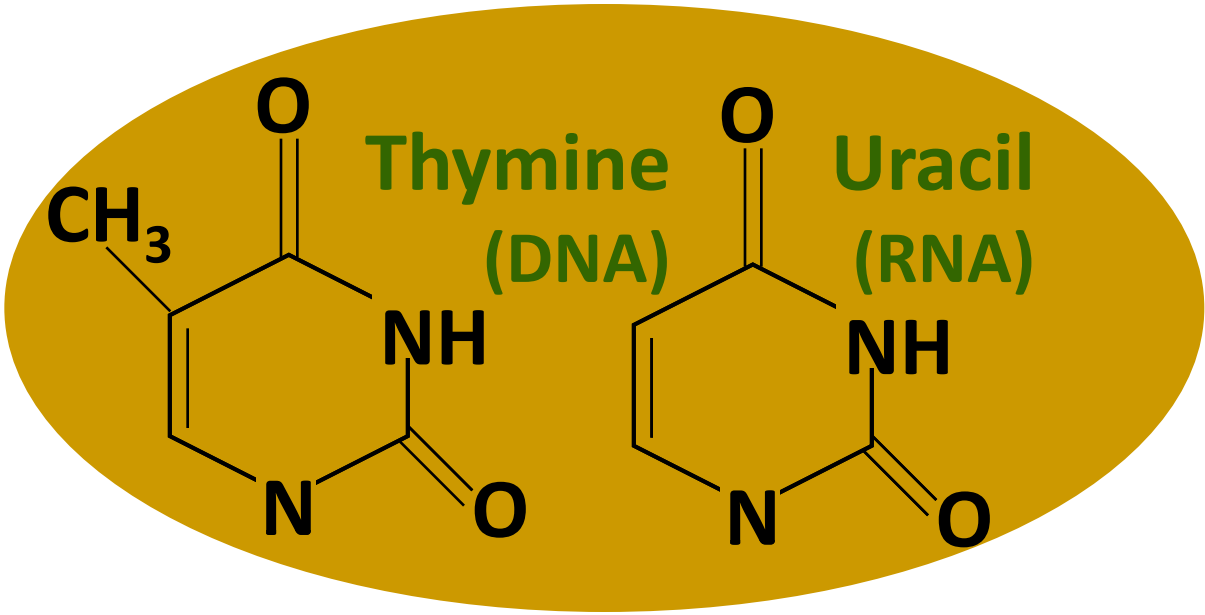
- **Deoxyribonucleic acid** (**DNA**) is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms and some viruses.
- The chemical and physical properties of DNA suit it for both replication and transfer of information. Each DNA molecule is a long two-stranded chain
- The strands are made up of subunits called nucleotides, each containing a sugar (deoxyribose), a phosphate group, and one of four nitrogenous bases, adenine, guanine, thymine, and cytosine, denoted A, G, T, and C, respectively

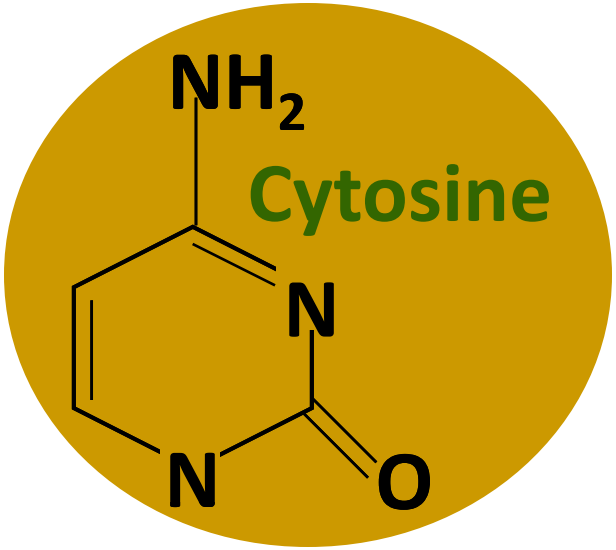
Purines





Pyrimidines





ADENINE

- IUPAC name — 9H-purin-6-amine
- Other names — 6-aminopurine

Properties

Molecular formula	$C_5H_5N_5$
Molar mass	135.127 g/mol
Melting point	360 – 365 °C

GUANINE

- IUPAC name 2-amino-1*H*-purin-6(9*H*)-one
- Other names 2-amino-6-hydroxypurine, 2-aminohypoxanthine,

Properties

Molecular formula $C_5H_5N_5O$

Molar mass 151.1261 g/mol

Appearance White amorphous solid.

Melting point 360°C (633.15 K) *deco.*

Boiling point Sublimes.

Solubility in water Insoluble.

CYTOSINE

- IUPAC name 4-amino-1H-pyrimidine

Properties

- Molecular formula $C_4H_5N_3O$
- Molar mass 111.300
- Melting point 320 - 325°C (decomp)

THYMINE

- IUPAC NAME5-Methylpyrimidine- 2,4(1*H*,3*H*)-dione

Properties

Molecular formula $C_5H_6N_2O_2$

Molar mass 126.11334 g/mol

Melting point 316 - 317 °C

URACIL (in RNA)

- IUPAC NAME Pyrimidine-2,4(1*H*,3*H*)-dione
- Other names Uracil, 2-oxy-4-oxy pyrimidine,
  2,4(1H,3H)-pyrimidinedione,
  2,4-dihydroxypryimidine,
  2,4-pyrimidinediol

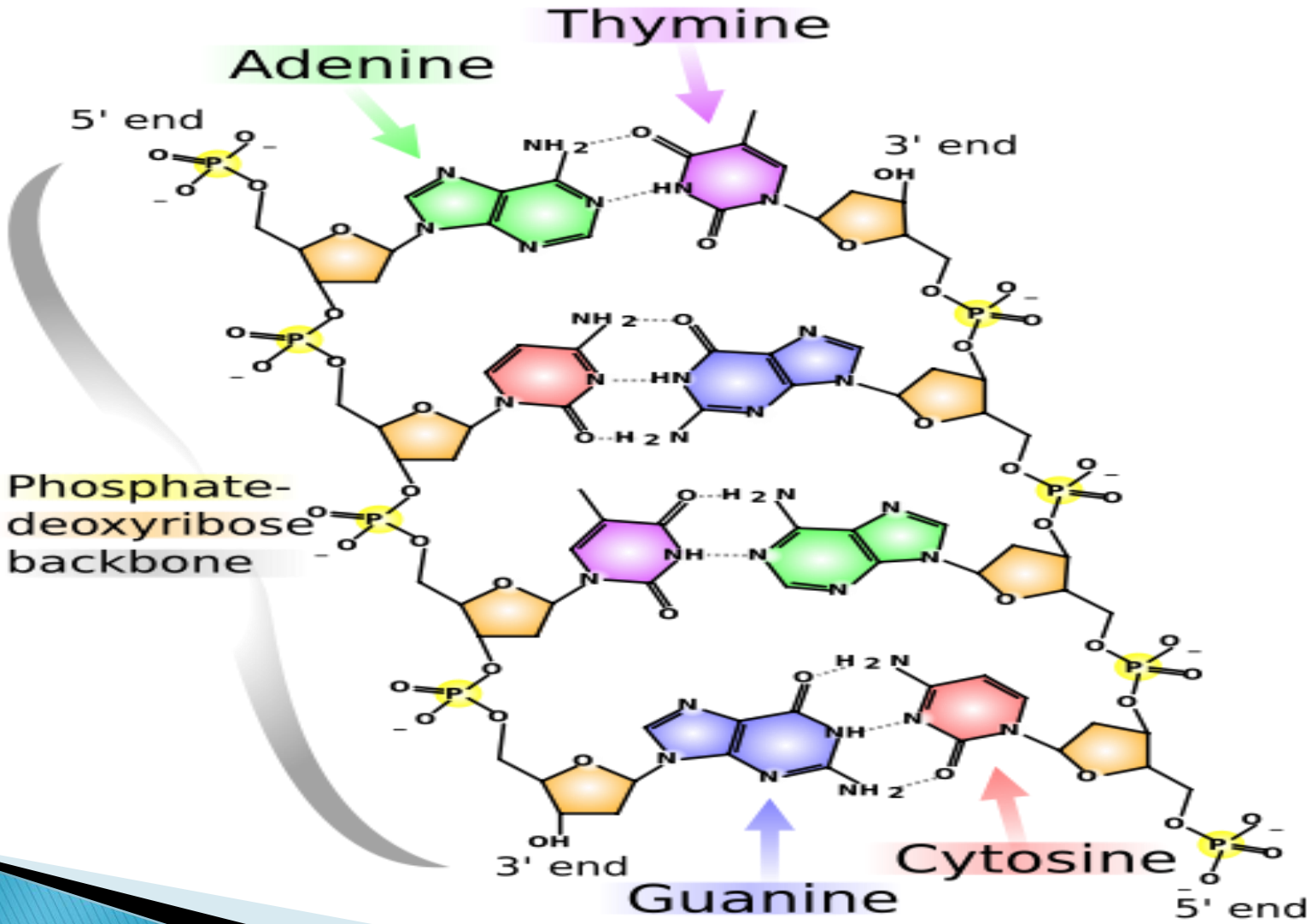
Properties

Molecular formula $C_4H_4N_2O_2$

Molar mass 112.08676 g/mol , Appearance Solid

Melting point 335 °C , Solubility in water Soluble

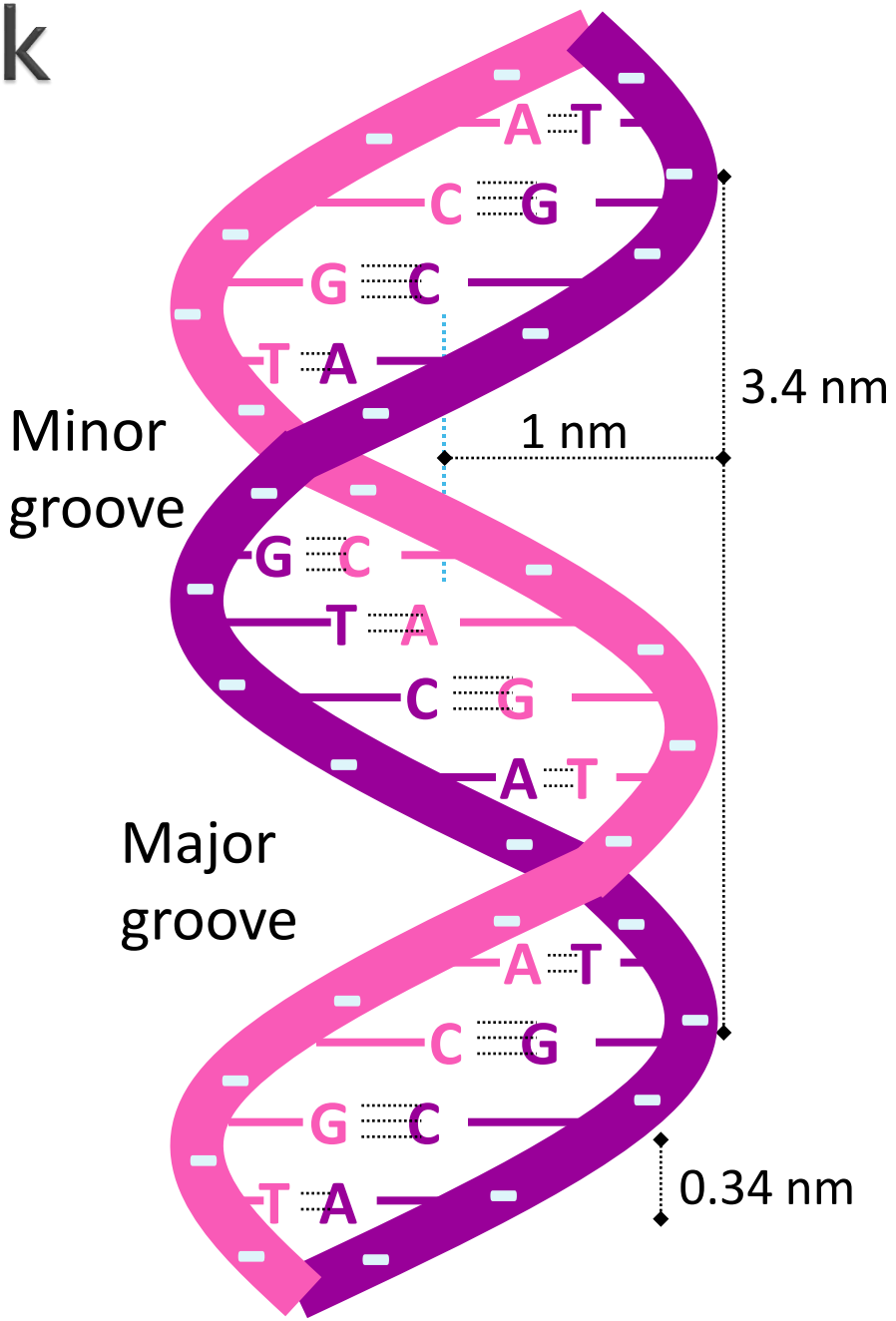
CHEMICAL STRUCTURE OF DNA



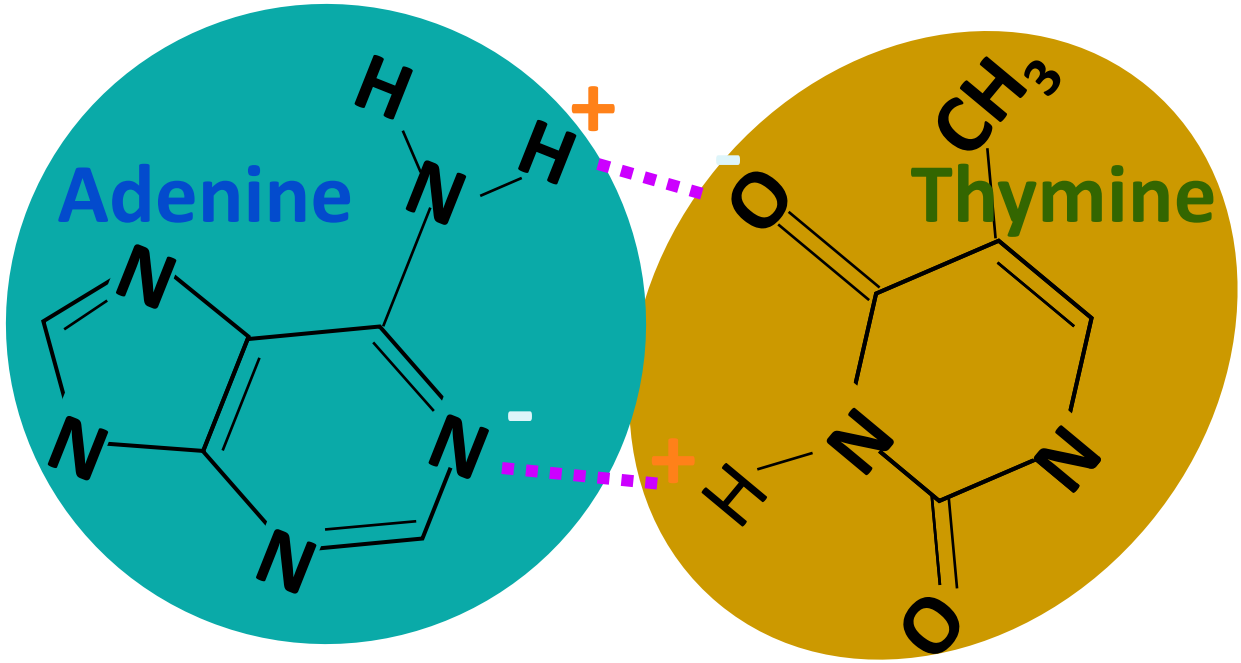
Contd..

- The backbone of the DNA strand is made from alternating phosphate and sugar residues. The sugar in DNA is 2-deoxyribose, which is a pentose sugar.
- The sugars are joined together by phosphate groups that form phosphodiester bonds between the 3rd and 5th carbon atoms of adjacent sugar rings. These asymmetric bonds mean a strand of DNA has a direction. In a double helix the direction of the nucleotides in one strand is opposite to their direction in the other strand.
- This arrangement of DNA strands is called antiparallel. The asymmetric ends of DNA strands are referred to as the 5′ and 3′ ends, with the 5' end being that with a terminal phosphate group and the 3' end that with a terminal hydroxyl group.

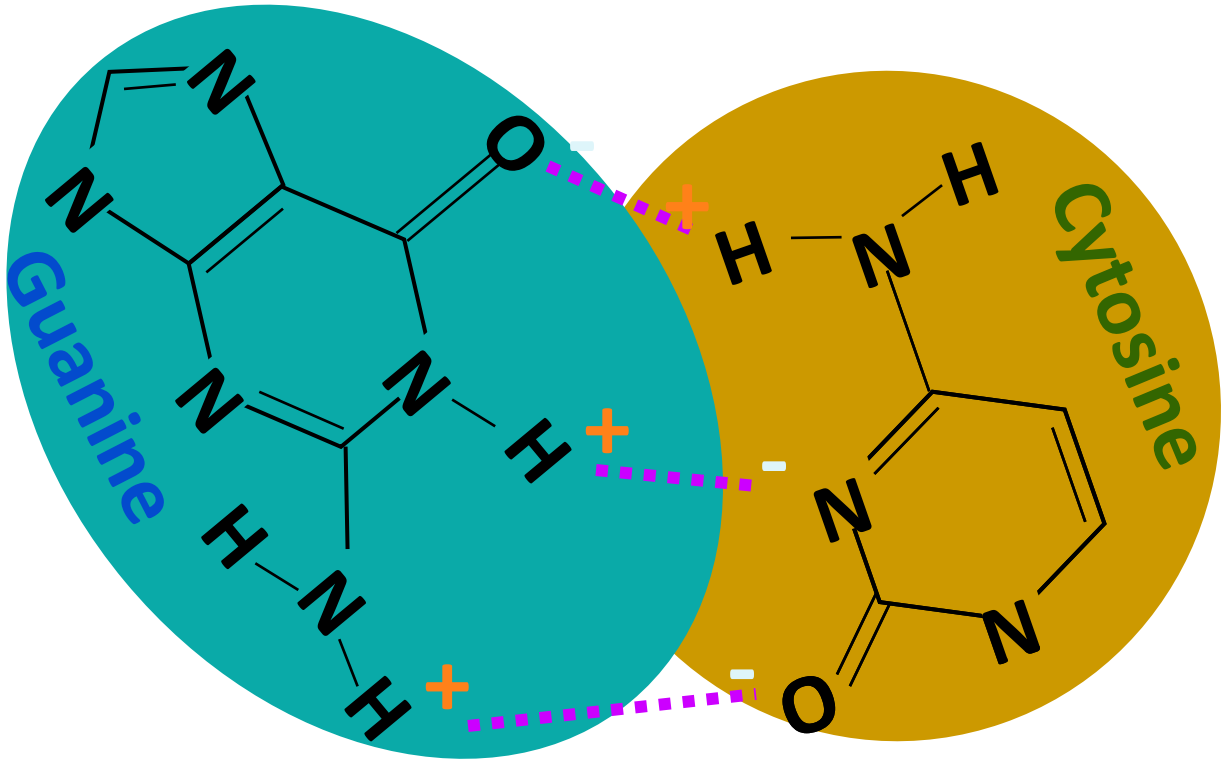
The Watson – Crick Model Of DNA



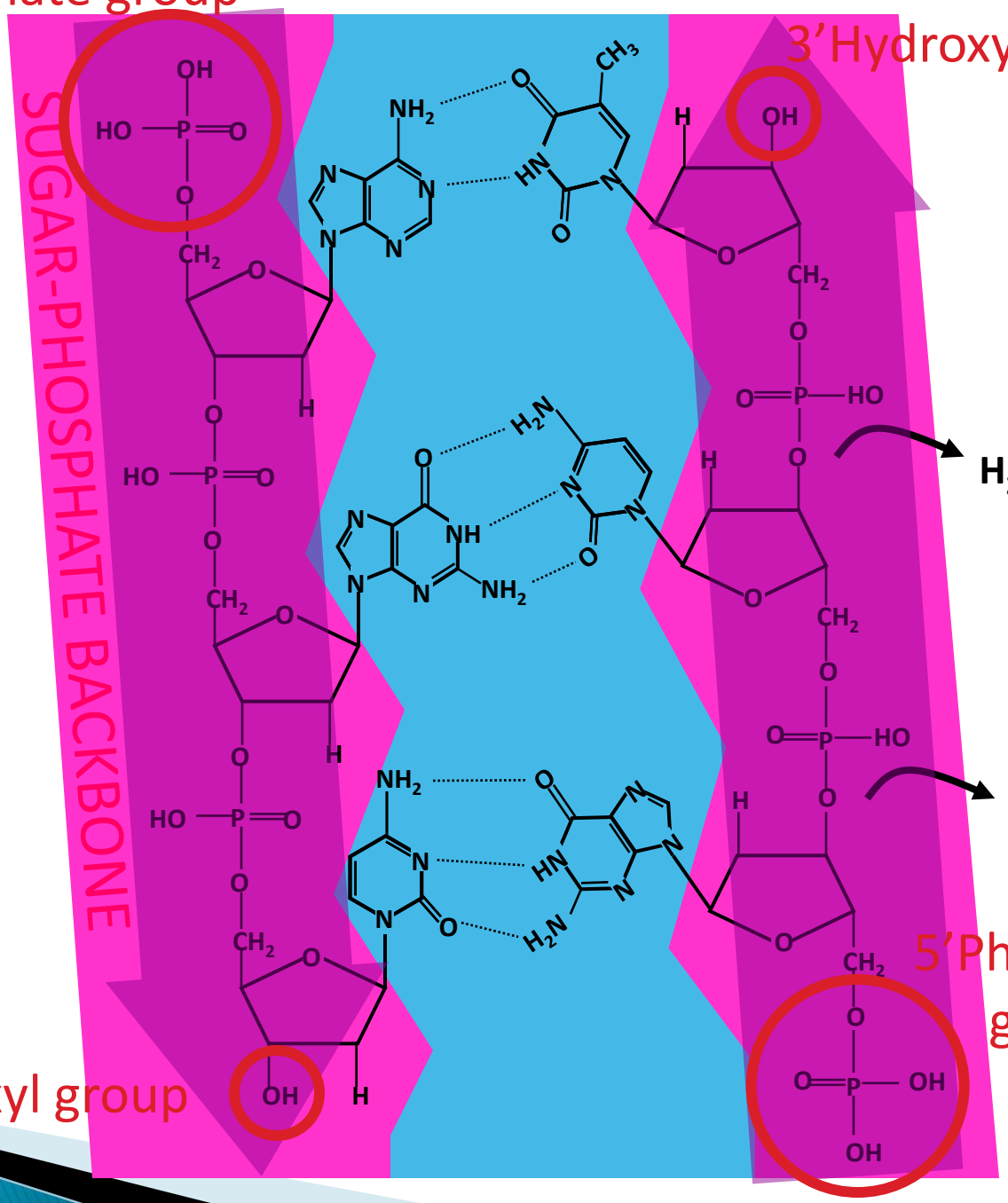
Base Pairing
Adenine And Thymine



Base Pairing
Guanine And Cytosine

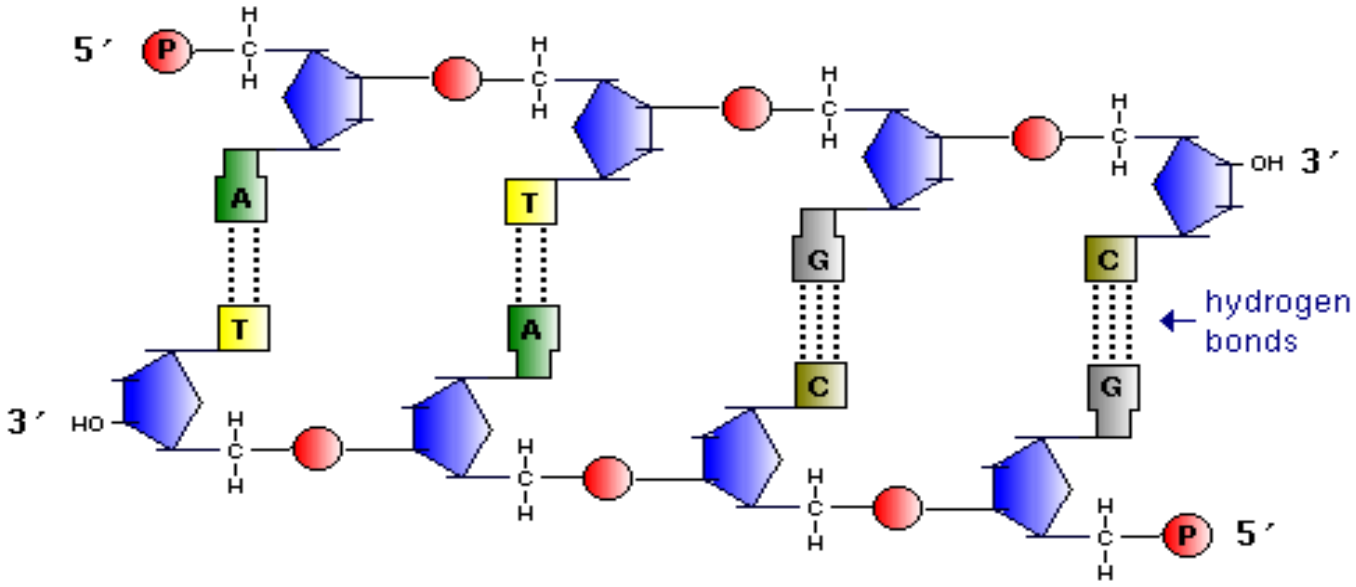


DNA


5’Phosphate group
3’Hydroxyl group
SUGAR-PHOSPHATE BACKBONE
H_2O
H_2O
5’Phosphate group
3’Hydroxyl group

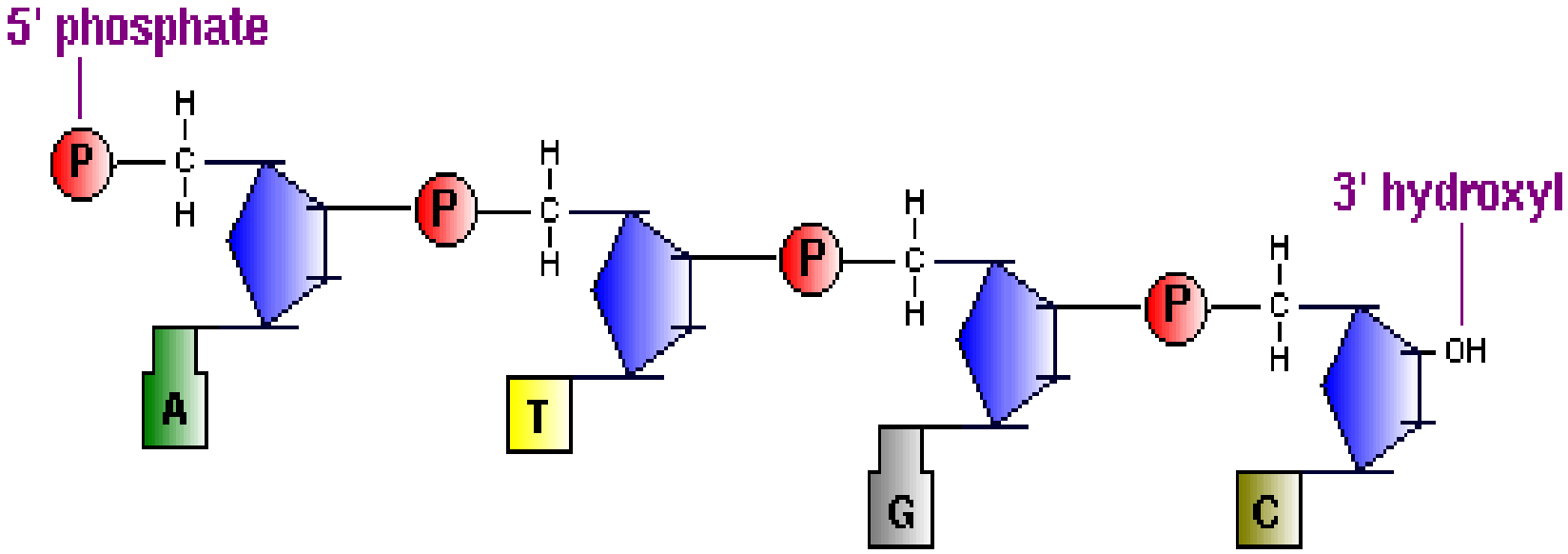
Base Pairing and Double Stranded Nucleic Acids

Most DNA exists in the famous form of a double helix, in which two linear strands of DNA are wound around one another. The major force promoting formation of this helix is complementary base pairing: A's form hydrogen bonds with T's (or U's in RNA), and G's form hydrogen bonds with C's.



The two strands of DNA are arranged antiparallel to one another: viewed from left to right the "top" strand is aligned 5' to 3', while the "bottom" strand is aligned 3' to 5'. *This is always the case for duplex nucleic acids.*

G-C base pairs have 3 hydrogen bonds, whereas A-T base pairs have 2 hydrogen bonds: one consequence of this disparity is that it takes more energy (e.g. a higher temperature) to disrupt GC-rich DNA than AT-rich DNA



A key feature of all nucleic acids is that they have two distinctive ends: the 5' (5-prime) and 3' (3-prime) ends. This terminology refers to the 5' and 3' carbons on the sugar. For both DNA (shown above) and RNA, the 5' end bears a phosphate, and the 3' end a hydroxyl group.

Another important concept in nucleic acid structure is that DNA and RNA polymerases add nucleotides to the 3' end of the previously incorporated base. Another way to put this is that nucleic acids are synthesized in a 5' → 3' direction

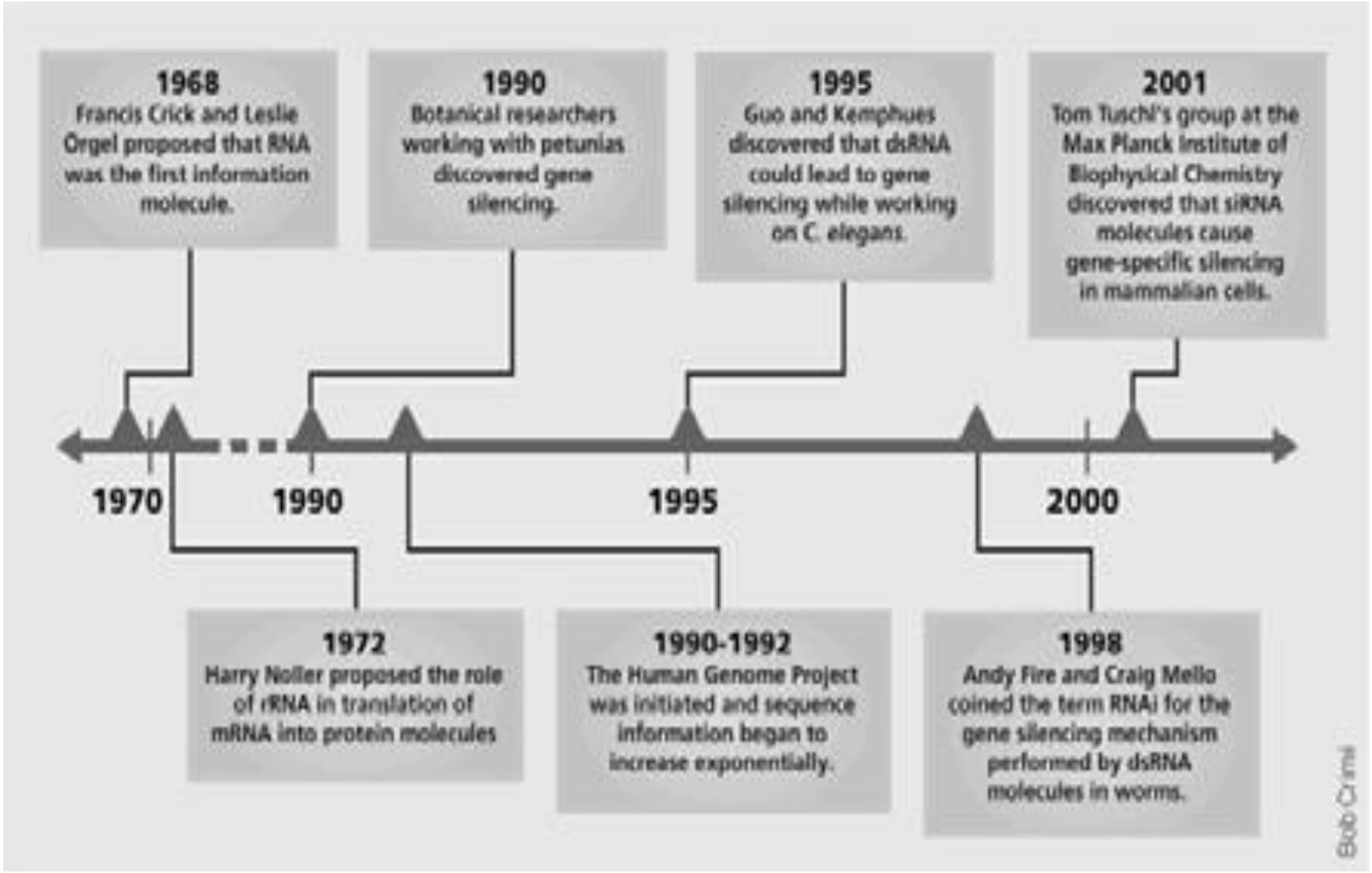
Abbr.	Base	Nucleoside	Nucleic Acid
A	Adenine	deoxyadenosine	DNA
		adenosine	RNA
G	Guanine	deoxyguanosine	DNA
		guanosine	RNA
C	Cytosine	deoxycytidine	DNA
		cytidine	RNA
T	Thymine	deoxythymidine (thymidine)	DNA
U	Uracil	uridine	RNA

MOLECULAR BIOLOGY
(22ZOOC23)

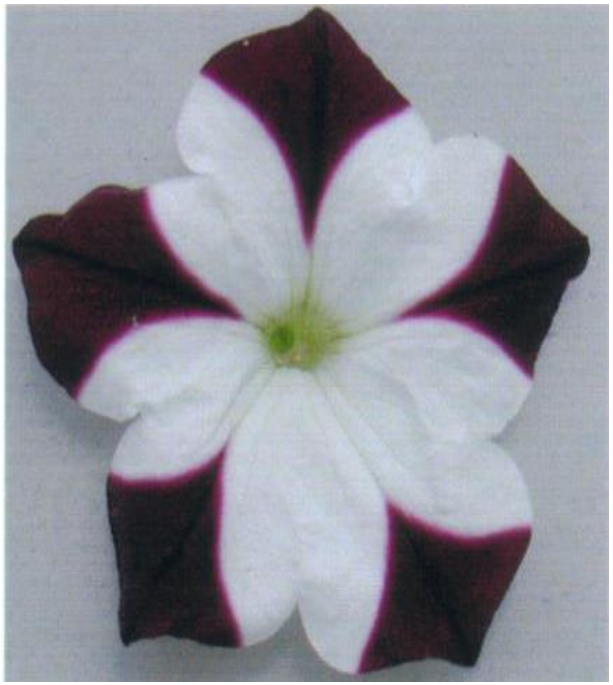
RNA Interference (RNAi)

---Regulation of Gene Expression by RNA

Timeline for RNAi Discoveries



Unexpected results in Petunia

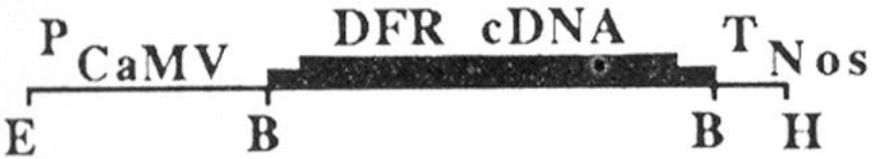


DFR construct introduced into petunia

CaMV - 35S promoter from Cauliflower Mosaic Virus

DFR cDNA – cDNA copy of the DFR mRNA (intronless DFR gene)

T Nos - 3’ processing signal from the Nopaline synthase gene

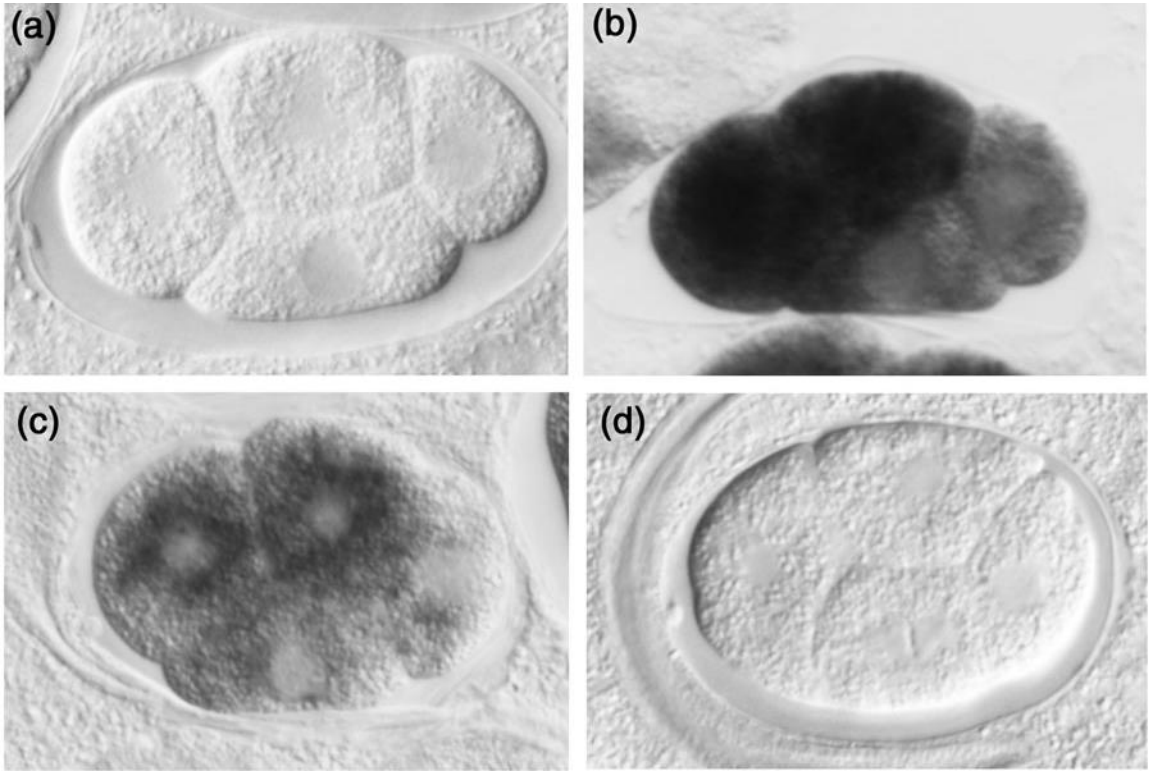


Flowers from 3 different transgenic petunia plants carrying copies of the chimeric DFR gene above. The flowers had low DFR mRNA levels in the non-pigmented areas.

Double-stranded RNA-induced RNA interference causes destruction of a specific mRNA in *C. elegans*

uninjected, no probe

uninjected, mex-3 probe



antisense mex-3 RNA, mex-3 probe

double-stranded mex-3 RNA injected, mex-3 probe

Guo, S. and Kemphues, K. J. *Cell* 81, 611-620 (1995)
Fire, A. et al. *Nature* 391, 809 (1998)

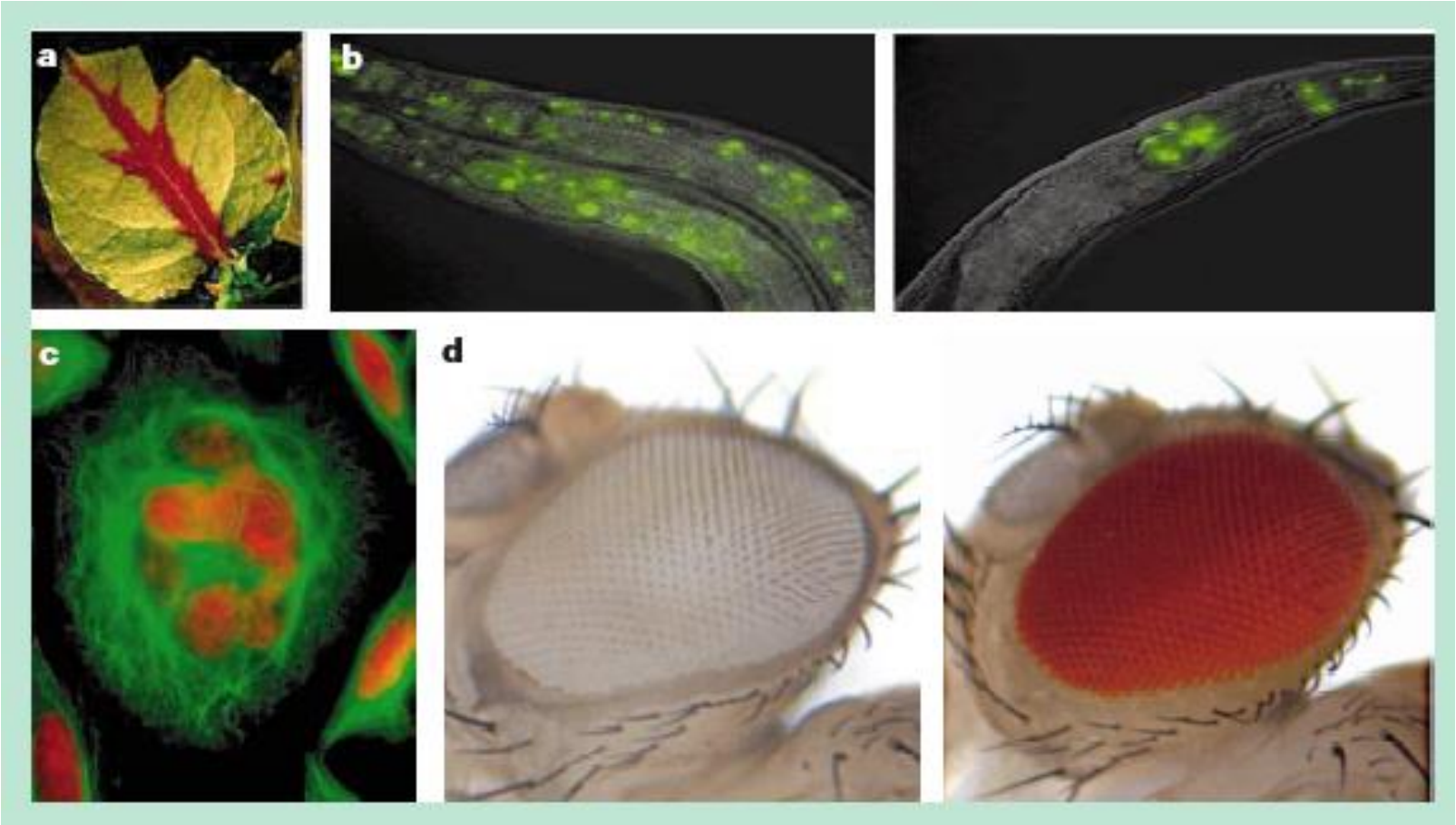
Key points of *C. elegans* experiment

- **substoichiometric amounts of dsRNA relative to the targeted mRNA are required to completely eliminate the mRNA (i.e. the dsRNA is catalytic)**
- **dsRNA is 10-100X better than antisense or sense RNA**
- **doesn't work if introns or promoters are targeted by the dsRNA**
- **doesn't interfere with transcription initiation or elongation (it is possible to target a single gene in an operon) (i.e. RNAi is a post-transcriptional phenomena)**
- **the targeted mRNA is degraded (i.e. it can't be detected by probes)**
- **dsRNA can cross cellular boundaries (i.e. there is a transport mechanism)**

RNAi works in other organisms

silencing of GFP in leaf veins

silencing of GFP in *C. elegans* nuclei



depletion of ORC6 results in multinucleated HeLa cells

depletion of White results in unpigmented Drosophila eyes

Hannon, G. J. *Nature* 418, 244-251 (2002)

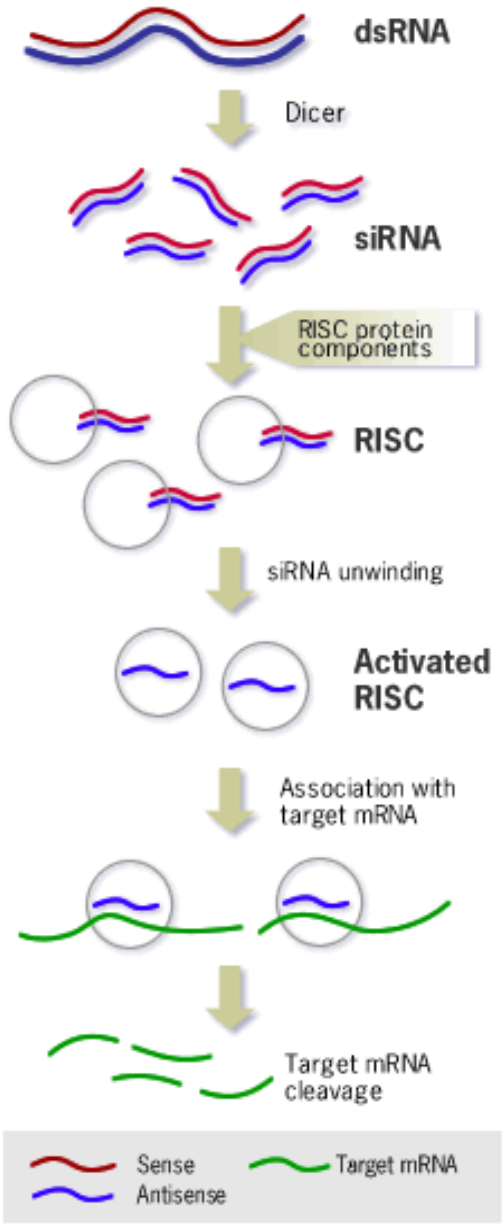
The Mechanism of RNA Interference (RNAi)

1. dsRNAs get processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer

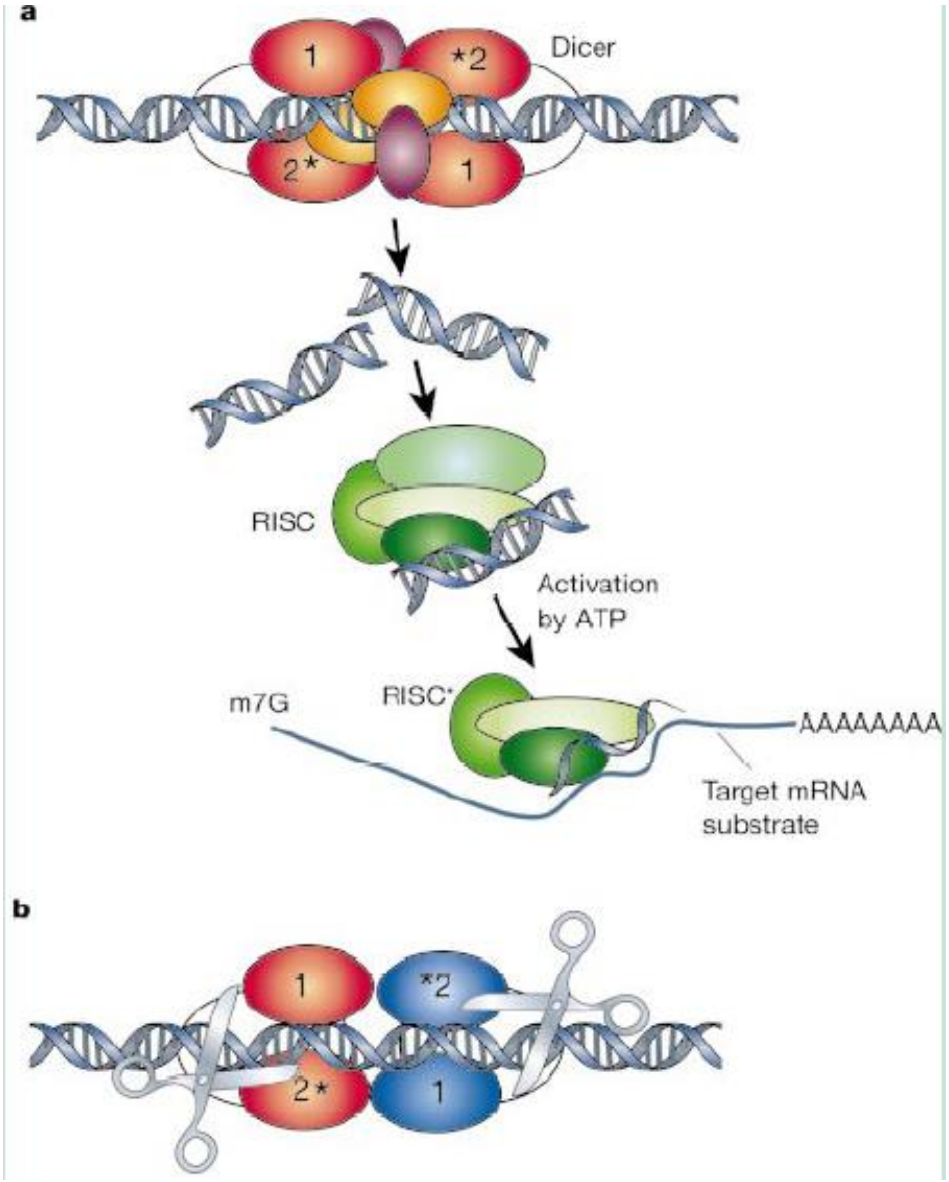
2.the siRNAs assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process

3.siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA

*** Long double-stranded RNAs (dsRNAs; typically >200 nt) can silence the target genes in organisms and cell types (e.g., worms, fruit flies, and plants)**



Dicer



Dicer contains 5 domains

- **2 catalytic RNase domains**
- **a C-terminal dsRNA binding domain (dsRBD).**
- **ATPase/RNA helicase domain**
- **a PAZ domain that is shared with Argonaute.**

Dicer is thought to work as a dimer

One of the catalytic sites in Dicer is defective

Thus, instead of cleaving from ~9-11 nt, like bacterial RNase III Dicer cleaves ~22 nt

generates siRNAs with 2-nt 3' Overhangs and phosphorylated 5' ends.

Argonaute

Argonaute proteins are found in all RISC and miRNA-containing ribonucleoprotein complexes and have been shown to be the catalytic site of mRNA cleavage in RISC [47].

Argonautes are ~100 kDa proteins characterized by conserved domains called PAZ and PIWI.

Argonautes can be organized by sequence into two subfamilies, Ago and Piwi, based on a higher degree of homology to either *Arabidopsis* AGO1 or to *Drosophila* Piwi.

The Ago group functions in RNAi and miRNA silencing pathways

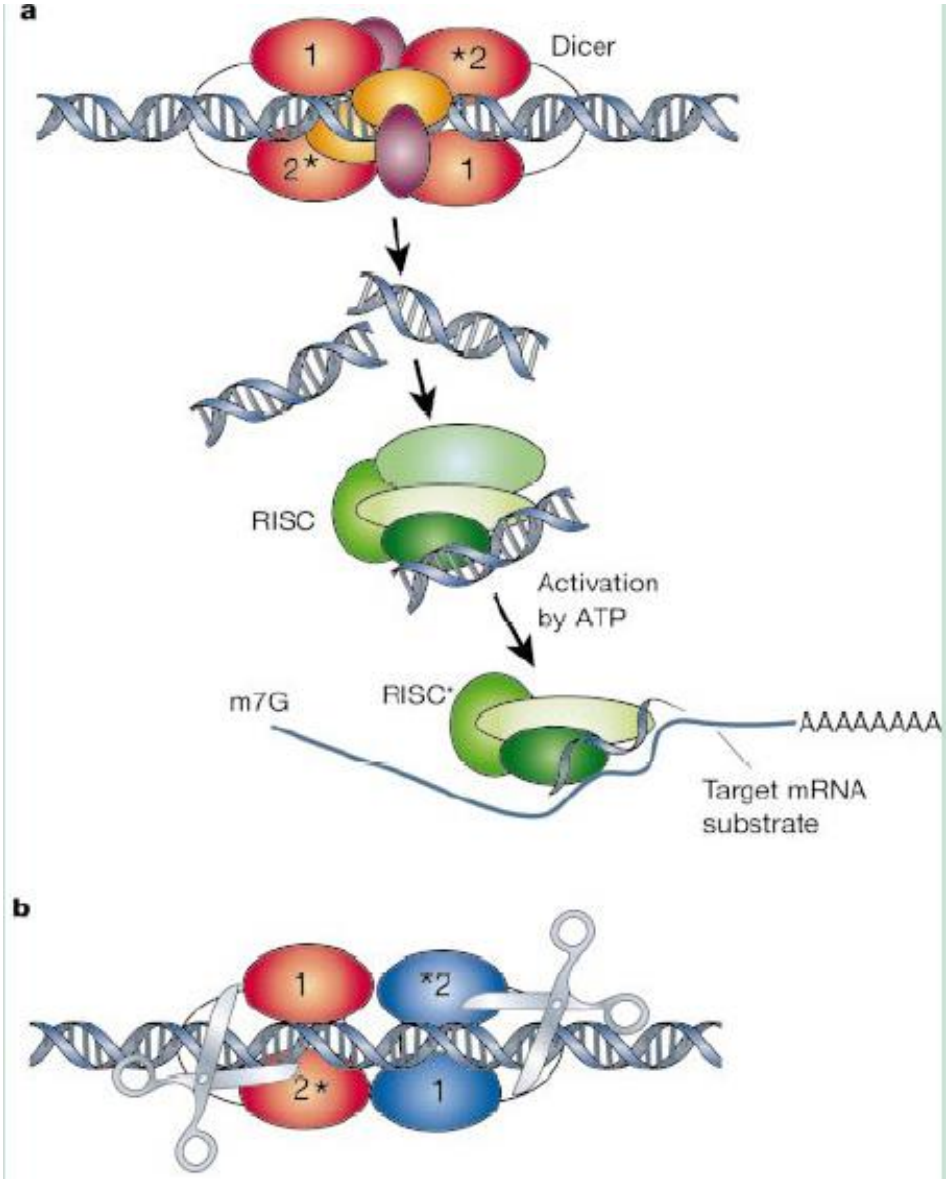
- **PAZ Domain**
  Conserved domain found in both Dicer and Argonaute. It is thought that the 3' end of the guide strand of an siRNA is in contact with the PAZ in RISC.

- **PIWI Domain**
  Conserved domain found in Argonaute and thought to be the catalytic site for mRNA cleavage. It is thought that the 5' end of the guide strand of the siRNA contacts the PIWI domain in RISC.

* **R2D2**

A small, dsRNA binding protein that works with Dicer in assembly of siRNA with RISC.

RISC

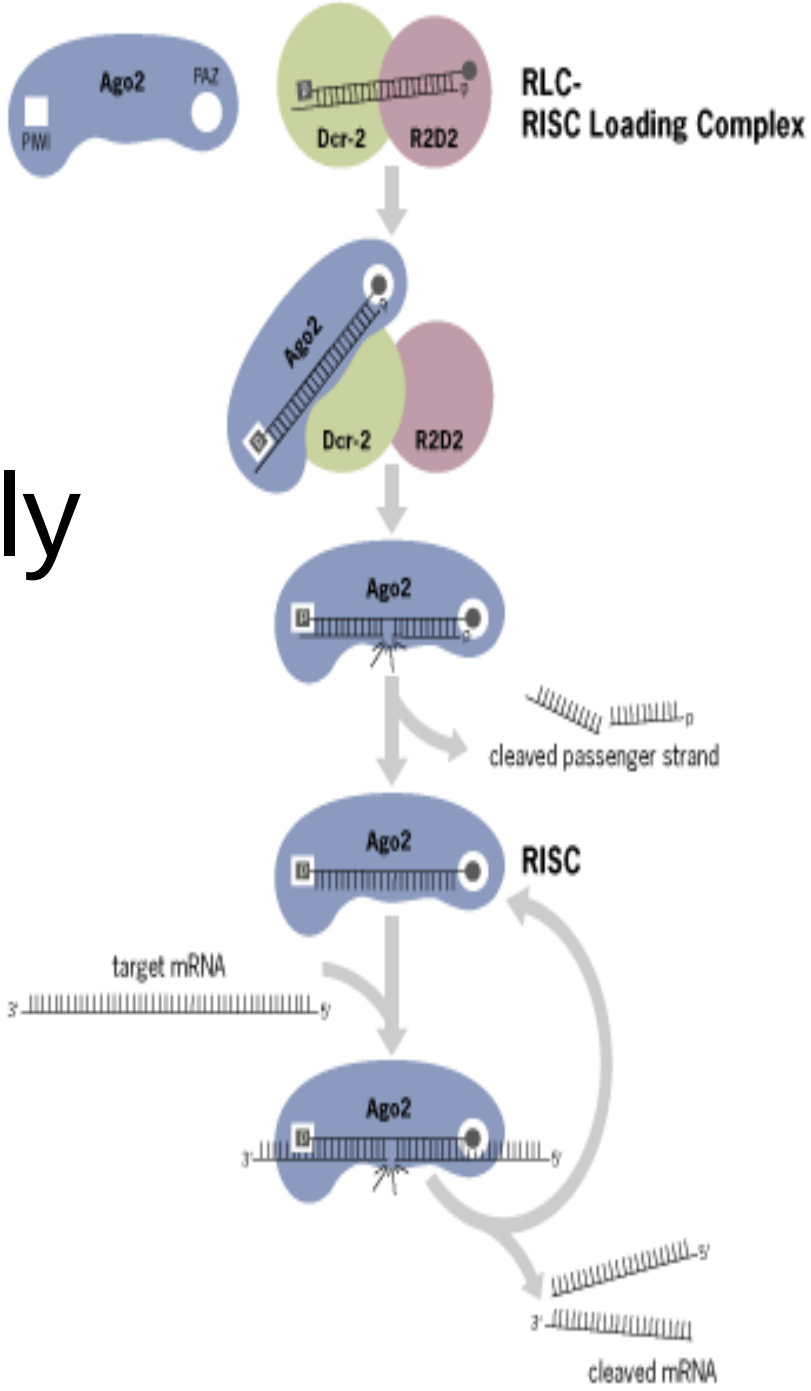


RISC contains at least 4 subunits

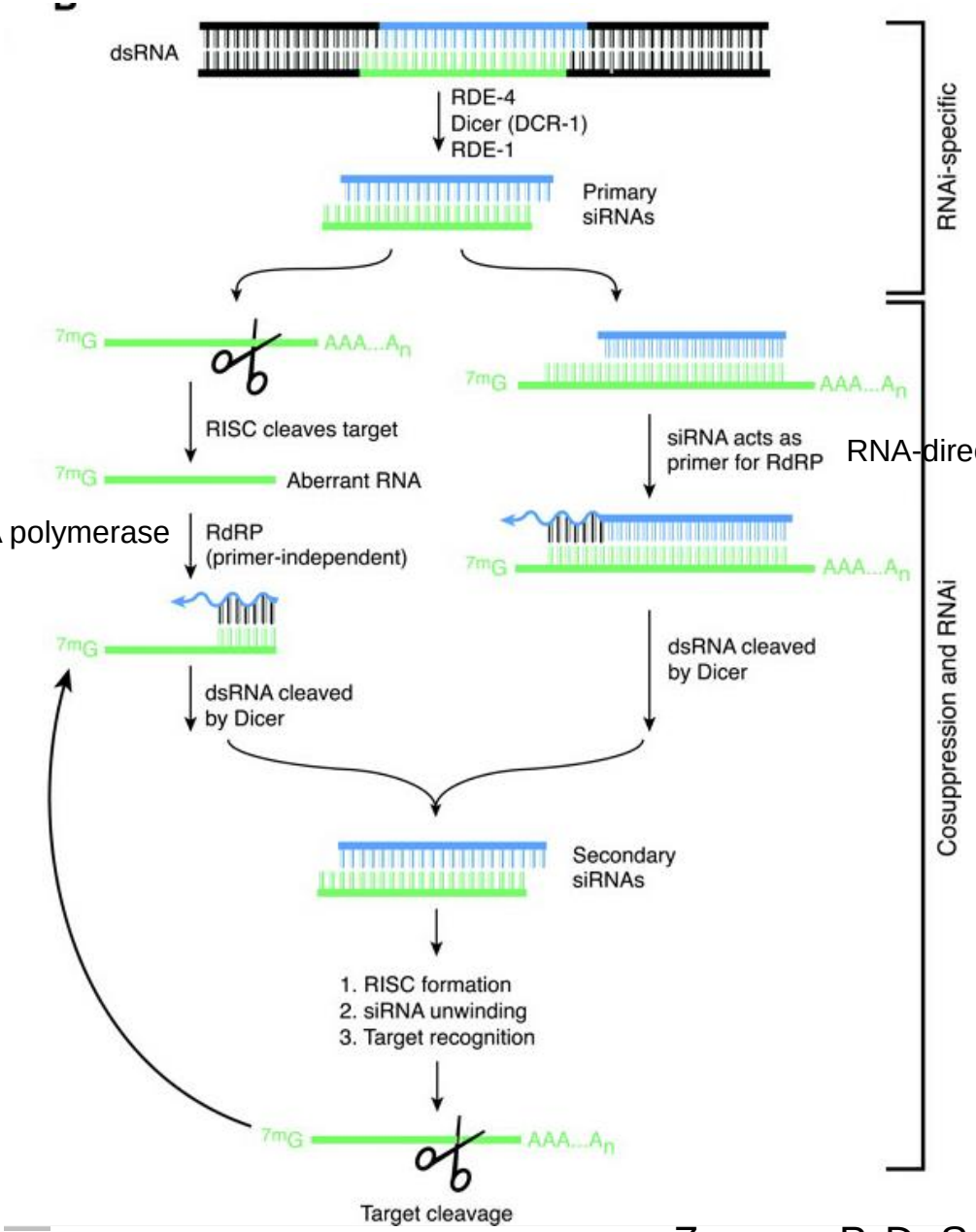
- **Argonaute (5 homologs in Dros.)**
- **dFXR (the Dros. homologue of human fragile X mental retardation protein)**
- **Vasa intronic gene (VIG)**
- **nuclease**

Activated RISC uses the unwound siRNA as a guide to substrate selection

RISC Assembly



A proposed model for amplification and spreading of silencing



Does not appear to occur in mammals, therefore the effects of siRNA are transient

Zamore, P. D. *Science* 296, 1265-1269 (2002)

Small RNA

miRNA

MicroRNA, a large class of evolutionarily conserved, noncoding, RNA originating from longer transcripts characterized by imperfect hairpin structures. miRNAs are 19–23 nt RNAs processed from pre-miRNA precursors by Dicer, the same enzyme that processes siRNAs.

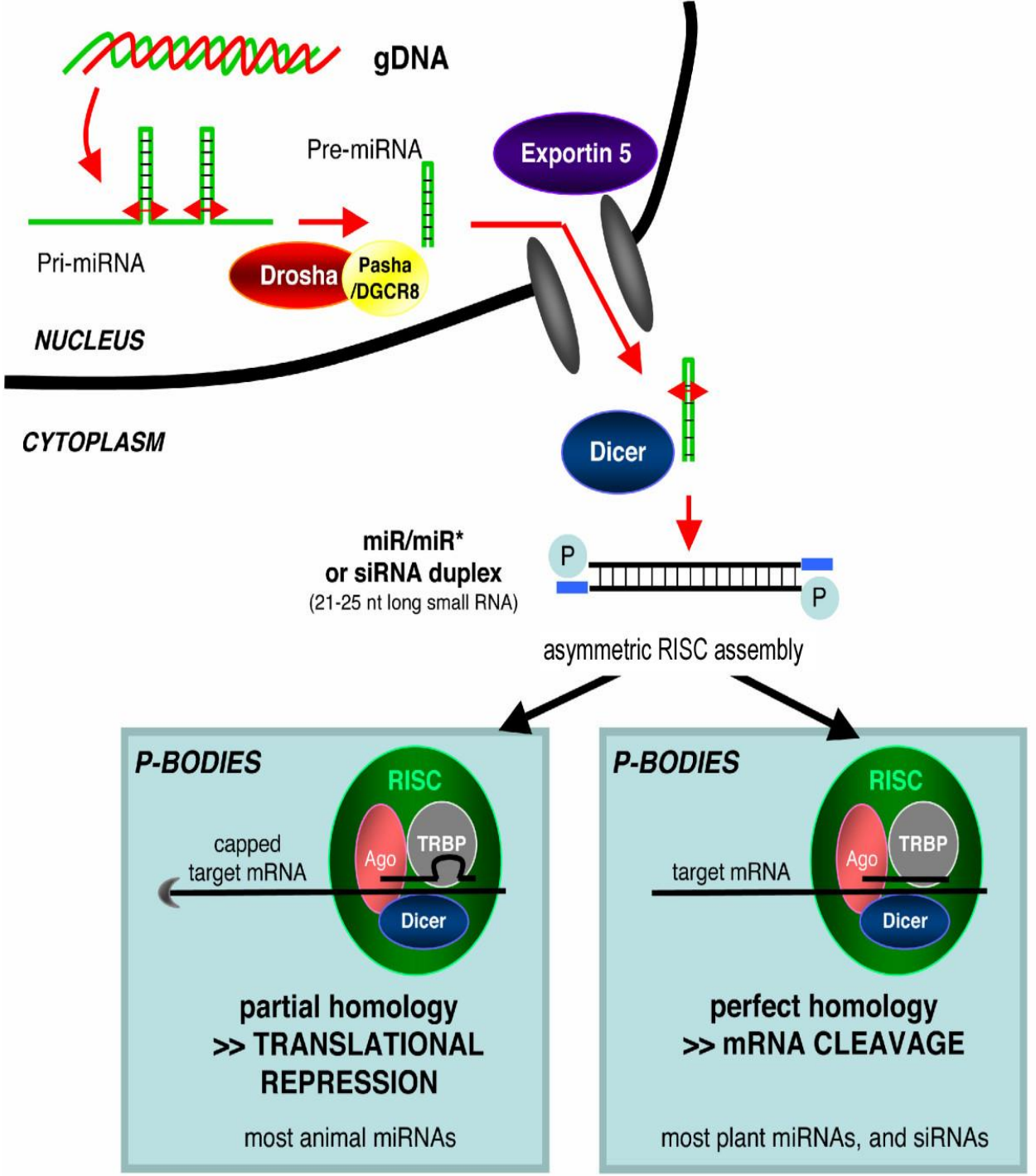
The first miRNAs to be discovered, lin-4 and let-7, were identified through loss-of-function mutations affecting control of postembryonic development in *C. elegans

rasiRNA

Repeat-associated siRNA, an endogenous form of siRNA that originates from repetitive elements within the genome.

- **siRNA**
  Short interfering RNA, siRNAs are 21–25 bp dsRNA with dinucleotide 3' overhangs that are processed from longer dsRNA by Dicer in the RNA interference pathway. Introduction of synthetic siRNAs can induce RNA interference in mammalian cells. siRNAs can also originate from endogenous dsRNA precursors.

- **shRNA**
  Short hairpin RNA. shRNAs are used in plasmid- or vector-based approaches for supplying siRNAs to cells to produce stable gene silencing. A strong promoter is used to drive transcription of a target sequence designed to form hairpins and loops of variable length, which are then processed to siRNAs by the cellular RNAi machinery.


gDNA
Pre-miRNA
Exportin 5
Pri-miRNA
Drosha
Pasha
/DGCR8
NUCLEUS
CYTOPLASM
Dicer
miR/miR*
or siRNA duplex
(21-25 nt long small RNA)
P
P
asymmetric RISC assembly
P-BODIES
RISC
capped
target mRNA
Ago
TRBP
Dicer
partial homology
>> TRANSLATIONAL
REPRESSION
most animal miRNAs
P-BODIES
RISC
target mRNA
Ago
TRBP
Dicer
perfect homology
>> mRNA CLEAVAGE
most plant miRNAs, and siRNAs

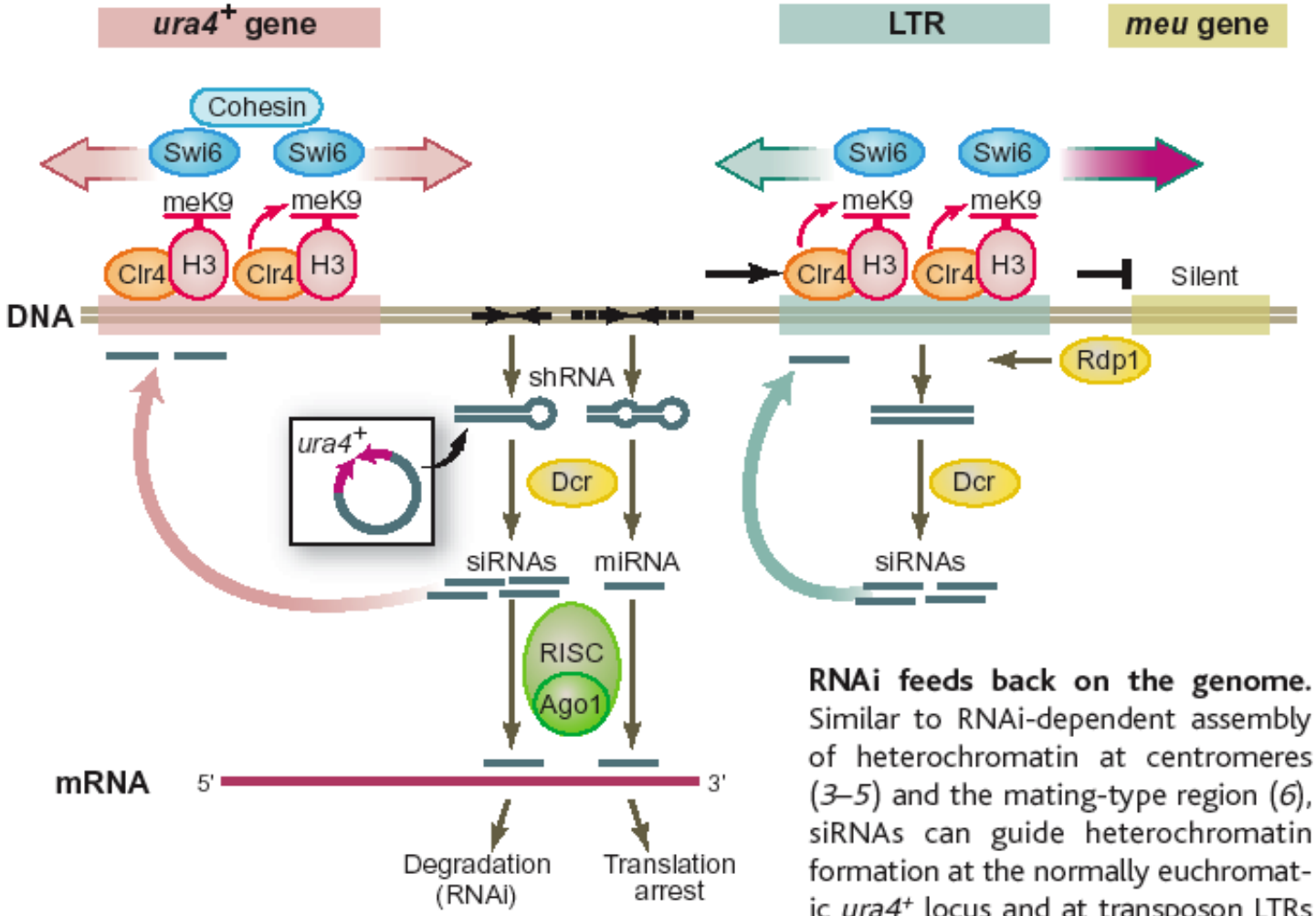
What is the endogenous biological function of the RNAi machinery?

(The RNAi machinery may provides a mechanism for responding to nucleic acid invaders.

It could stabilize the genome by sequestering repetitive sequences, such as transposons, in heterochromatin making them unavailable for recombination events that would lead to chromosomal translocations)

RNAi can silence gene expression through other mechanisms:

Histone methylation occurs at loci homologous to the siRNA target



RNAi feeds back on the genome. Similar to RNAi-dependent assembly of heterochromatin at centromeres (*3–5*) and the mating-type region (*6*), siRNAs can guide heterochromatin formation at the normally euchromatic *ura4*+ locus and at transposon LTRs

Matzke, M. and Matzke, A. J. M. *Science* 301, 1060-1061 (2003)
Schramke, V. and Allshire, R. *Science* 301, 1069-1074 (2003)

Figure 4: Methods for Producing siRNAs and shRNAs

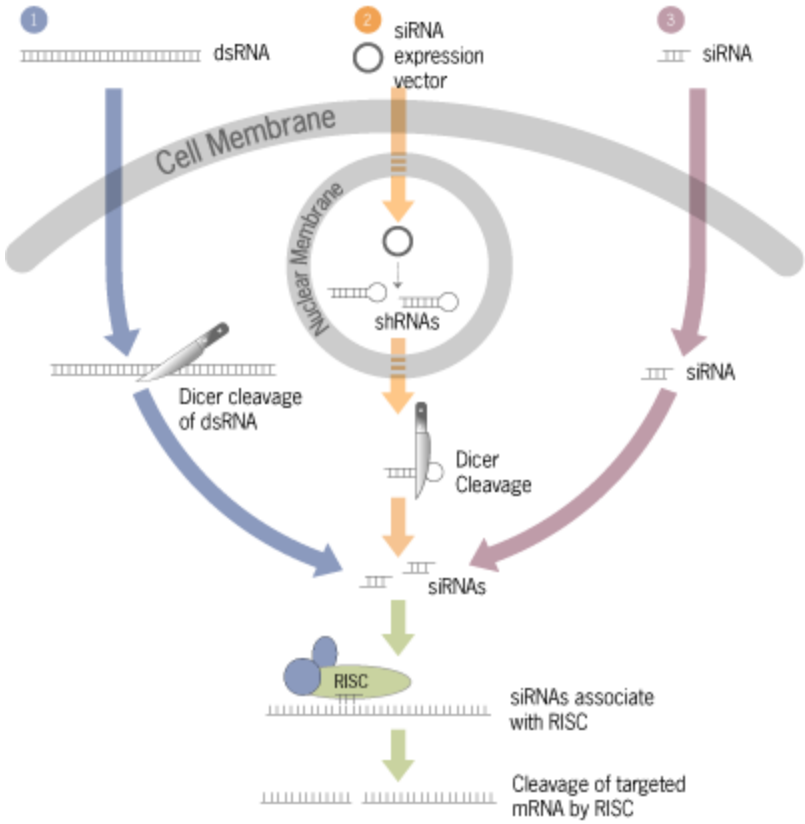
	Chemical synthesis	In vitro transcription (IVT)	Dicer/RNase III digestion	Expression vector
Procedure	1. Order pre-designed siRNAs online OR 1. Design siRNA and order custom siRNAs online	1. Design siRNAs 2. Order DNA oligonucleotides 3. Perform IVT reaction 4. Anneal 5. Treat with DNase and ssRNA- specific RNase 6. Perform column purification	1. Create transcription template 2. Perform IVT reaction 3. Anneal 4. Clean up dsRNA 5. Digest with 6. Dicer/RNase III 7. Purify	1. Design siRNAs 2. Order DNA oligonucleotides 3. Clone into linearized vector 4. Isolate clones 5. Verify sequence 6. Perform large scale prep
Requirements	Two 21-mer RNA oligonucleotides	Two 29-mer DNA oligonucleotides	Transcription template (200–800 bp region flanked by promoters)	Two 55–60-mer DNA oligonucleotides
Ability to fluorescently label siRNA	Yes	Yes	Yes	No (however some vectors code for Green Fluorescent Protein)
Useful for long-term studies?	No	No	No	Yes, using certain vectors with antibiotic resistance markers
Ambion Product	***Silencer*® Pre-designed siRNA** ***Silencer*® Validated siRNA** ***Silencer*® Control siRNA** ***Silencer*® siRNA Libraries** ***Silencer*® CellReady™ siRNA Libraries** **Custom siRNA Synthesis**	***Silencer*® siRNA Construction Kit**	***Silencer*® siRNA Cocktail Kit (RNase III)**	**p*Silencer*™ siRNA Expression Vectors** **p*Silencer*™ adeno 1.0-CMV System** **p*Silencer*™ 5.1 Retro System**

Three Ways to Trigger the RNAi Pathway.

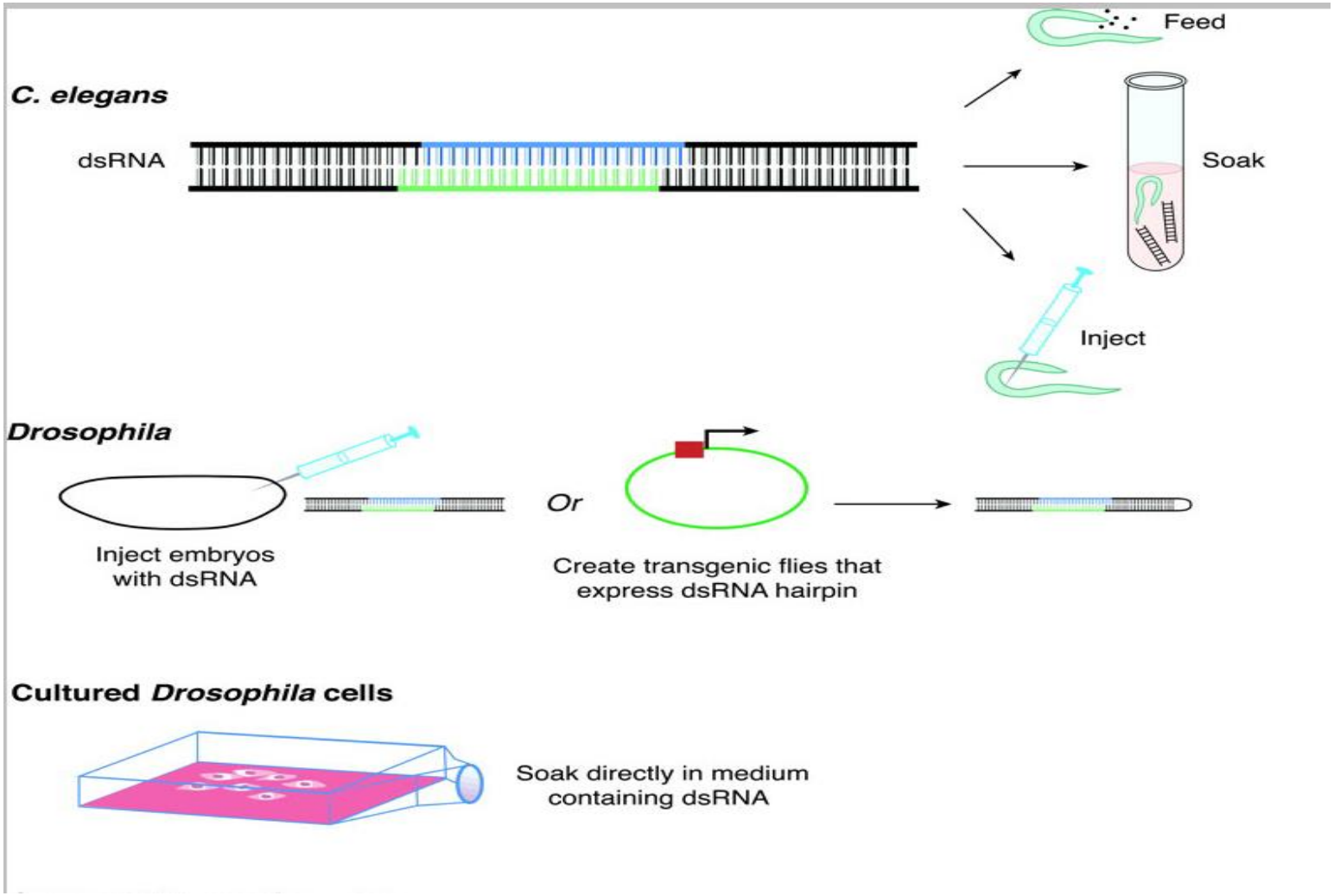
- **1.In non-mammalian systems, when double-stranded RNA (dsRNA; usually longer than 30 bp) is introduced into cells.**

- **2. In mammalian systems, RNAi can be triggered by DNA based expression vectors designed to express short hairpin RNA (shRNA) molecules**

- **3.synthetic short interfering RNA (siRNA) molecules.**

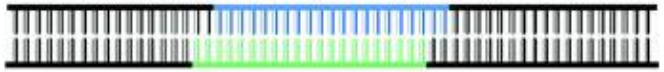


EFFECTIVE DELIVERY METHOD



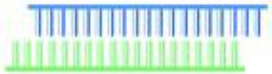
Cultured Mammalian cells

Embryonic cultured cells or oocytes or early embryos



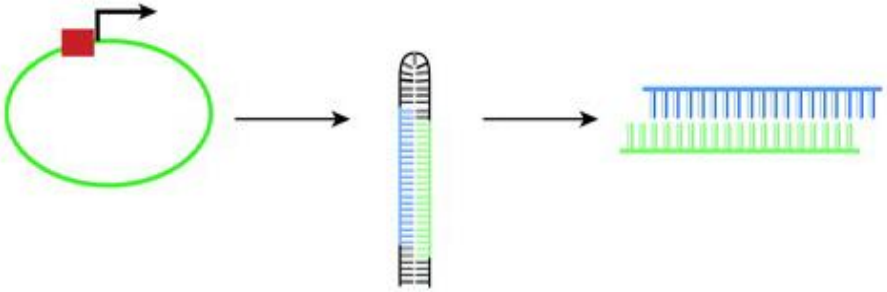
Transfect or inject dsRNA

Differentiated cultured cells or whole organisms (in vivo)



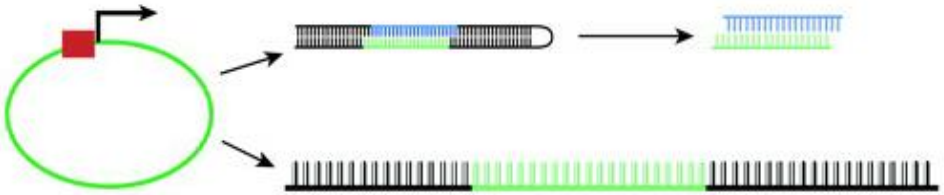
Transfect synthetic siRNA

Or



Transfect plasmids expressing miRNA precursor-like stem-loop to generate siRNA in cells

Plants



Overexpress dsRNA or a dsRNA hairpin from a transgene

Overexpress single-stranded RNA from a transgene or virus to trigger cosuppression

Most immortalized, adherent cell lines

Transfect siRNAs with:
transfection agents (lipid mediated or amine based)

Primary cells, suspension cells, and other "difficult to transfect" lines

Electroporate siRNAs

ASSAY

- The simplest and arguably the best assay for siRNA validation and transfection optimization relies on qRT-PCR to measure target transcript levels in gene-specific siRNA treated cells versus negative control siRNA treated cells.

- most researchers wish to determine the extent of knockdown at the protein level. A simple method for isolating total RNA and protein from the same sample. RNA isolated with the kit can be used for RT-PCR, array analysis, Northern blotting, or other analytical technique

- The protein lysate is compatible with Western blotting and, since native protein is recovered in most cases, enzymatic assay.

- also isolates small RNA species, including siRNAs. The ***mir*Vana miRNA Detection Kit** provides a way to quantitate siRNA levels in cell populations, and can also be used to examine target mRNA levels.

APPLICATIONS OF RNAi

- Determine gene function
- Pathway analysis
- Identify and validate drug targets
- Study gene redundancy
- Functional screening

MOLECULAR BIOLOGY

(22ZOOC23)

GENE THERAPY

(Viral vectors)

INTRODUCTION

- Gene therapy can be defined as the delivery of nucleic acid into the cell for the purpose of acquiring new features or restoration of physiologic status.
- The idea that disorders can be treated by genes arose in the 1960s, when the mechanism of cell transformation by SV40 virus and papovaviruses was described.
- Gene therapy enables **modification** of cell by the replacement of non-functional or missing gene, suppression of another gene, or induction of cell death as in the case of oncologic diseases.

- There are approximately 1000 clinical trials conducted or in progress applying gene therapy applications. The majority of these, almost 70%, are based on viral vectors.
- Two **important features of viruses** have made them attractive to use.
    - Viral vectors have generally proved **efficient vehicles** for gene delivery to target cells/tissue, a critical aspect of achieving therapeutic efficacy.
    - Another important factor has been the establishment of **high level transgene expression**.

- **Monogenic diseases** (sickle cell anaemia, cystic fibrosis, Huntington's disease, Duchenne muscular dystrophy) **and** **age-related disorders** (obesity, type II diabetes, heart and renal failure) can be treated by retrovirus-mediated gene therapy, but (retro)viral vectors are most frequently used in cancer gene therapy.

History of gene therapy and retroviral vectors

- The work of ***Howard M. Temin*** performed on **Rous sarcoma virus (RSV)** is the fundamental part in the research of retroviruses and retroviral vectors. He discovered that specific genetic mutations could be inherited as a result of viral infection.

- In the 1970s, specific viral genes involved in the transformation were discovered. The SRC and other (proto-)oncogenes with cellular origin were described.

- Pioneer work was performed on avian-infectious alpha-retroviral Rous sarcoma virus, **Moloney murine leukaemia virus (MoMLV)** belonging to gamma-retroviruses was initially used for the preparation of **therapeutic vector**, and until now, MoMLV-derived constructs along with human immunodeficiency virus (HIV)-derived vectors are most frequently used.

Comparison between retroviral and other viral and non-viral systems

- There are **two systems** for the delivery of transgene into the cell – **viral** and **non-viral (**safe; less effective**)**.

- The nonviral - polymer nanoparticles, lipids, calcium phosphate, electroporation/nucleofection or biolistic delivery of DNA-coated microparticles.

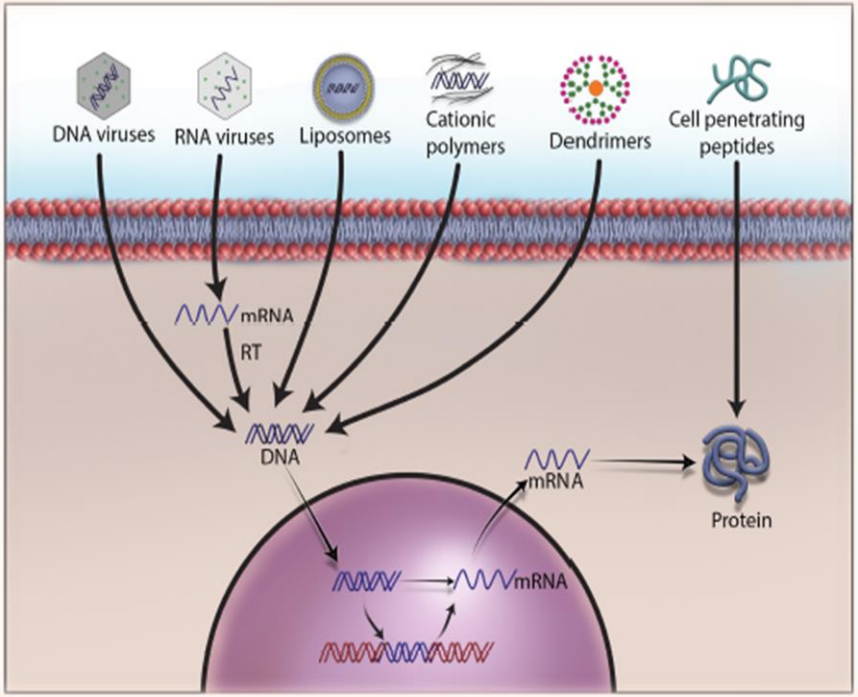
- There are several categories of viral vectors. Mainly categorized - two main types of viral vectors depending on whether the DNA is integrated into chromatin of the host cell or not.

1. Retroviral vectors derived from **gamma-retroviruses or lentiviruses** persist in the nucleus as integrated provirus and reproduce with cell division.
2. Other types of vectors (e.g. those derived from **herpesviruses or adenoviruses**) remain in cell in the episomal form.

Different methods to deliver therapeutic DNA and proteins to target cells

Non-viral gene delivery methods have many advantages over viral vectors in gene therapy.

They do not cause immunogenicity and carcinogenicity, and can deliver a large size of therapeutic DNA efficiently with a low price tag.



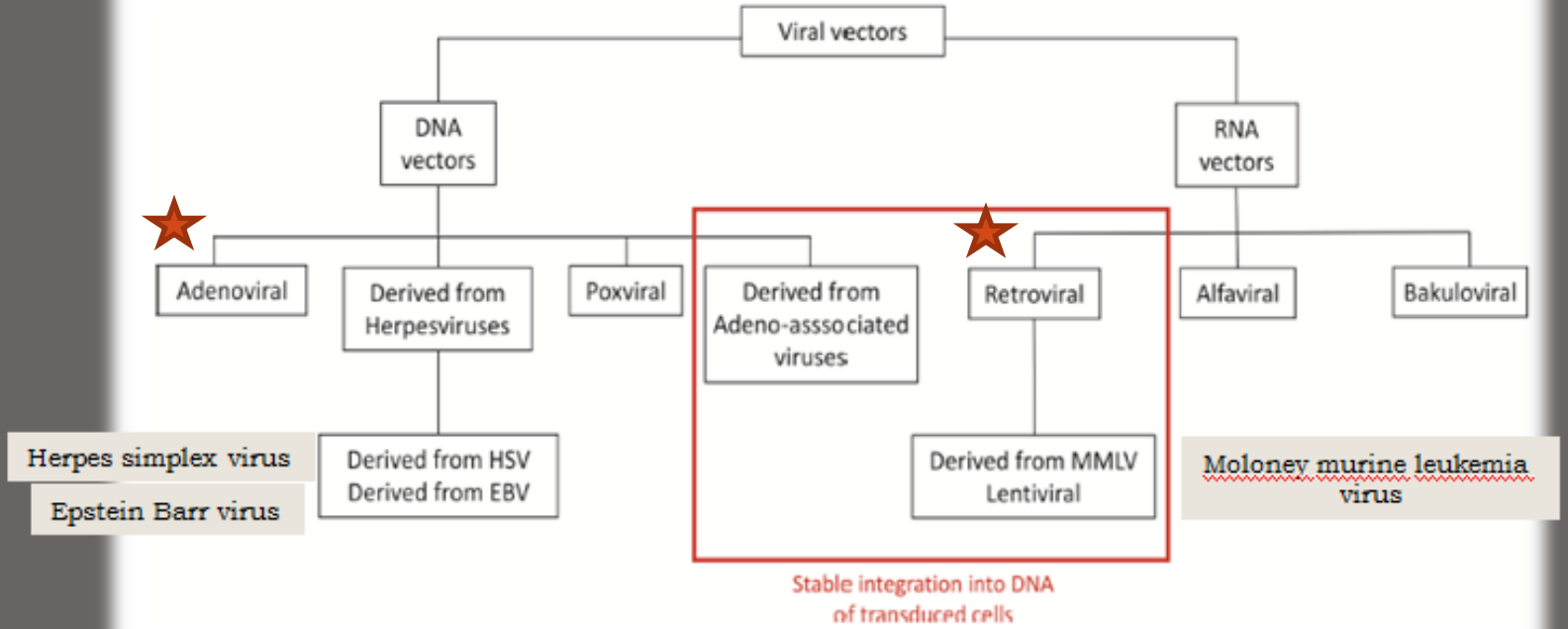


Figure 1. Overview of viral vectors

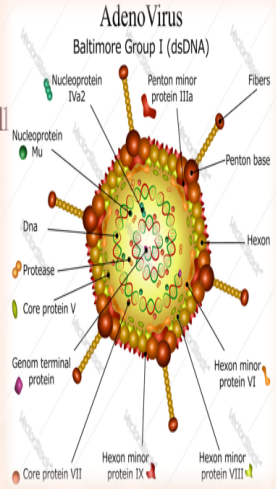
Adenoviral vectors (AV)

- **Adenoviral vectors (AV)** are very popular and have been used for several decades.

  - Non-enveloped dsDNA viruses, relatively resistant to chemical and physical agents, which enable them to persist out of host cells and make the work in laboratory easier in comparison to enveloped RNA viruses. They are often used in cancer gene therapy as replication-defective or replication-competent vectors. They infect proliferating as well as non-dividing cells.

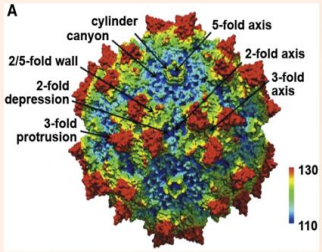
- These **dsDNA vectors** have the capacity to harbor approximately **8 kb of foreign DNA** and are efficiently packaged in mammalian cell lines such as HEK3 (Human Embryonic Kidney) cells.

- In general, adenoviral vectors are considered **safe**. Since they **do not integrate into host DNA**, the transduction is transient. The drawback is their immunogenicity.



- **Adeno-associated vectors (AAV)** share with retroviral vectors the ability to integrate into host DNA.
- The integration of AAV into the host genome generally occurs in a defined area of chromosome 19.
- Recombinant vectors lack this characteristic and the risk of insertional mutagenesis exists.
- The chromosomal integration provides a means of long-term transgene expression, which has been observed in many different tissues such as liver, muscle, retina, and the central nervous system (transduce dividing and non-dividing cells).
- Transduced cells are minimally immunogenic.

Variable region	Capsid structure
I, III, VII, IX	2/5-fold wall
II	Top of the 5-fold channel
IV, V, VIII	Top of the 3-fold protrusion
VI, VII	Base of the 3-fold protrusion



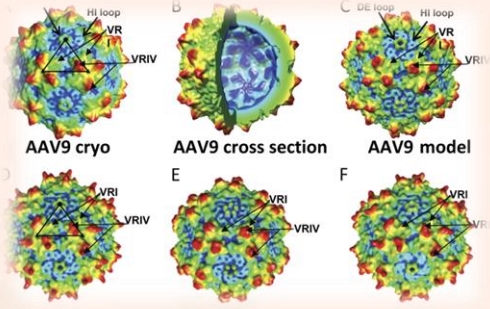
▪ **Several serotypes of AAV exist**, each showing substantial differences in their properties of cell transduction and transgene expression patterns.

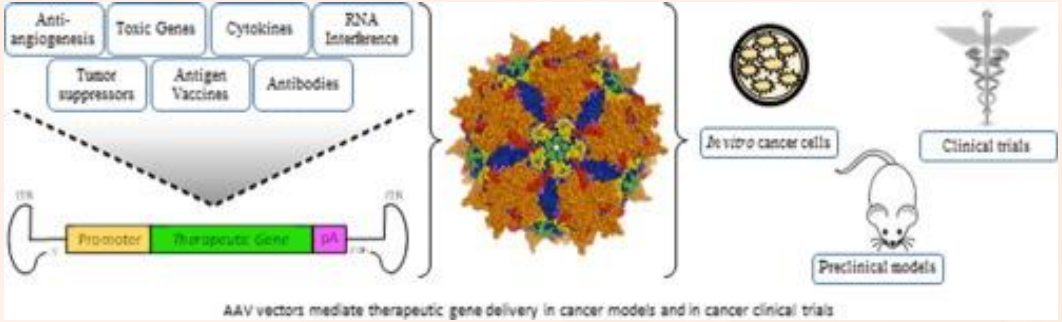
✓ **AAV1 serotype** is suitable for expression in skeletal muscle and retina cells, whereas the AAV2 shows more moderate but long-term expression.

✓ **AAV5 serotype** generates high expression levels in neuronal and lung cells.

✓ **AAV7 and AAV8 serotypes**, isolated from rhesus monkeys, have demonstrated 100-fold higher transgene expression levels of factor IX (FIX) in liver cells than any other AAV serotype.

AAV7 and AAV8 vectors were also expressed in the portal vein of LDL receptor-deficient mice, which led to normalization of the serum lipid levels and protection against development of severe atherosclerosis.





AAV vectors mediate therapeutic gene delivery in cancer models and in cancer clinical trials

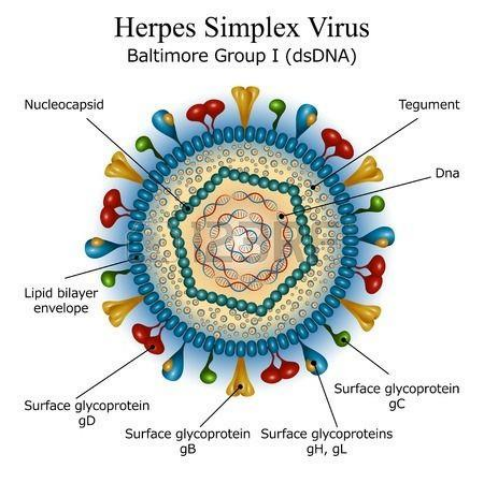
- **Herpes viruses** are relatively large (152 kb DNA genome), complex enveloped dsDNA viruses.

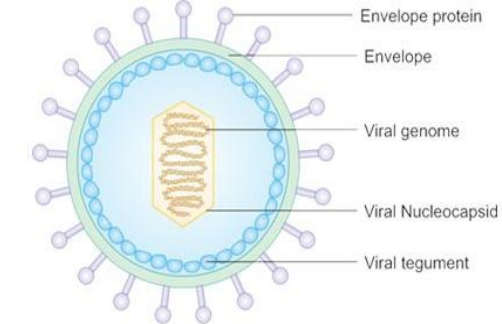
- Large genome size allows for the introduction of large foreign DNA insertions and permits modifications, including deletions of non-essential genes such as ICP0, ICP4, ICP22, and ICP47,
which has generated vectors with substantially reduced cytotoxicity.
- The vectors have been prepared from Herpes simplex type 1 virus, Epstein–Barr virus or cytomegalovirus.
- They are less immunogenic in comparison to adenoviruses.
- The transduction is transient. They can also establish a latent life-cycle, which means that they can present a life-long persistence in host organisms, and therefore provide long-term transgene expression.

- Herpes virus-derived vectors are preferentially used in vaccination and they have a strong neurotropism, which should make them suitable for treatment of neurodegenerative diseases.

- Oncolytic HSV vectors have been evaluated in many different animal tumor models including glioma, melanoma, breast, prostate, colon, ovarian, and pancreatic cancer.

- Improved efficacy was obtained by combination treatment with radiotherapy or chemotherapy.

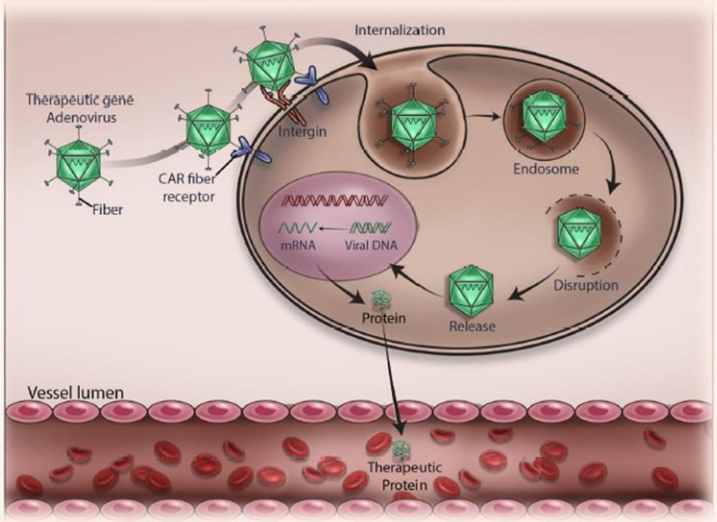




Epstein Bar Virus

Mechanism of adenovirus-mediated delivery of a therapeutic DNA.

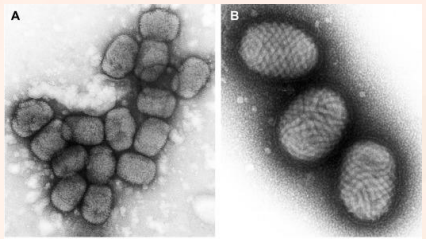
Upon infection, adenovirus delivers the encapsulated therapeutic DNA into the cytoplasm of the target cells. Various stages of viral gene delivery, viz cell attachment, internalization, endocytosis, uncoating, transcription and translation of the therapeutic protein

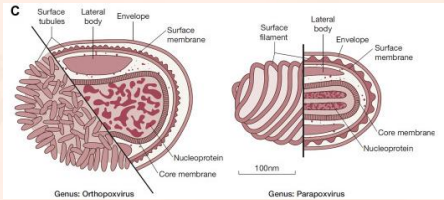


- **Poxviruses** are the most complex viruses.

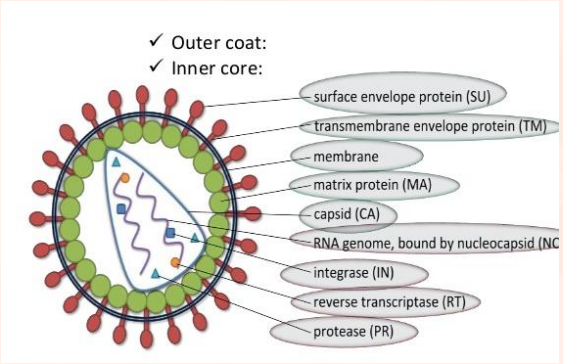
- Their major advantage lies in the huge cloning capacity. Upto 25 kbp of foreign DNA can be cloned into vaccinia-derived vectors.

- Similarly to herpes virus-derived vectors, they are popular in the preparation of vaccines including cancer immunotherapy.





Retroviral vectors



Retroviruses are relatively complex enveloped RNA viruses with diploid ssRNA genome (possessing reverse transcriptase activity, which enables them to integrate as DNA copies in the host genome).

- Viral RNA is reversibly transcribed and integrated in the form of provirus. They very effectively cooperate with enzymes of the host cell, and they use it for their own replication and long-term expression of viral proteins. The entry of virus into the host cell is receptor-dependent.
- Many types of retroviruses (**bovine leukaemia virus (BLV), Rous sarcoma virus (RSV), lentiviruses (LVs) and spumaviruses (SVs)**) were used for preparation of vectors.

- Due to the chromosomal integration long-term transgene expression can be established.
- Retroviruses show a relatively broad host range although non-diving cells are not susceptible. In this context, retrovirus vectors have been considered attractive for the treatment of brain tumors as the neuronal cells are not affected.

Eg: Experiments in cell cultures and animal models have demonstrated efficient retrovirus-mediated gene transfer and killing of glioma cells.

- Early gene therapy studies were to a large extent based on retrovirus vectors, typically applying murine leukemia virus (MLV). The most popular vectors are constructs based on MoMLV and HIV.

➢ The most exciting and impressive **gene therapy application of retrovirus vectors** so far has definitely been the treatment of infants with the SCID-X1 (severe combined immunodeficiency) disease.

The immune deficiency is caused by a mutation in the "γc gene" and was corrected by infusion of hematopoietic stem cells *ex vivo transduced with a retrovirus* vector carrying the healthy gene. The results were encouraging, with 9 out of 10 patients cured.

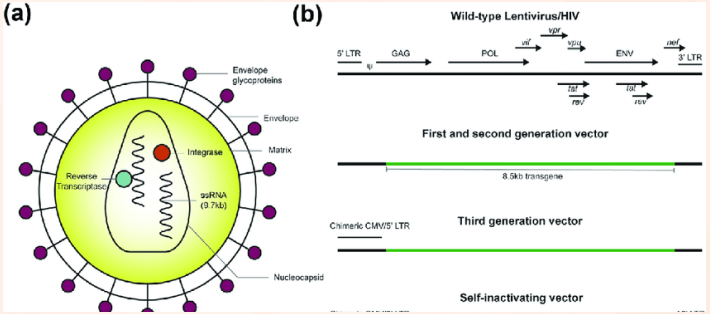
Unfortunately, a T-cell leukemia developed in 2 patients after 3 years (due to the integration of the therapeutic gene in an oncogenic region of the chromosome).

A **modified vector (MND-IL-2R)** has been developed for SCID-X1, which contains the IL-2 γc DNA and the Moloney murine leukemia virus (MoMLV) enhancer substituted with the corresponding region from the myelo-proliferative sarcoma virus (MPSV).

▪ **Lentiviruses** are also retroviruses, but have some unique features that give them a broader application range in gene therapy.

- They share many common features with retroviruses such as the dsRNA genome, relatively good capacity of packaging foreign genes and the means to integrate into the host genome.

- However, different from other retroviruses, lentiviruses can **efficiently transduce both dividing and non-dividing cells**, which make them attractive for applications in neurobiology too.



Eg:

1. Lentivirus-based delivery of the glial cell line-derived neurothrophic factor (GDNF) has been evaluated in a **primate model for Parkinson's disease**.

2. Introduction of the mouse albumin promoter in an HIV-1-based vector resulted in a highly liver-specific expression of parenchymal and nonparenchymal liver cells in **SCID mice**.

Long-term transgene expression could be established in human CD34+ cells from an HIV-1 based vector in a SCID mouse model.

Eg:

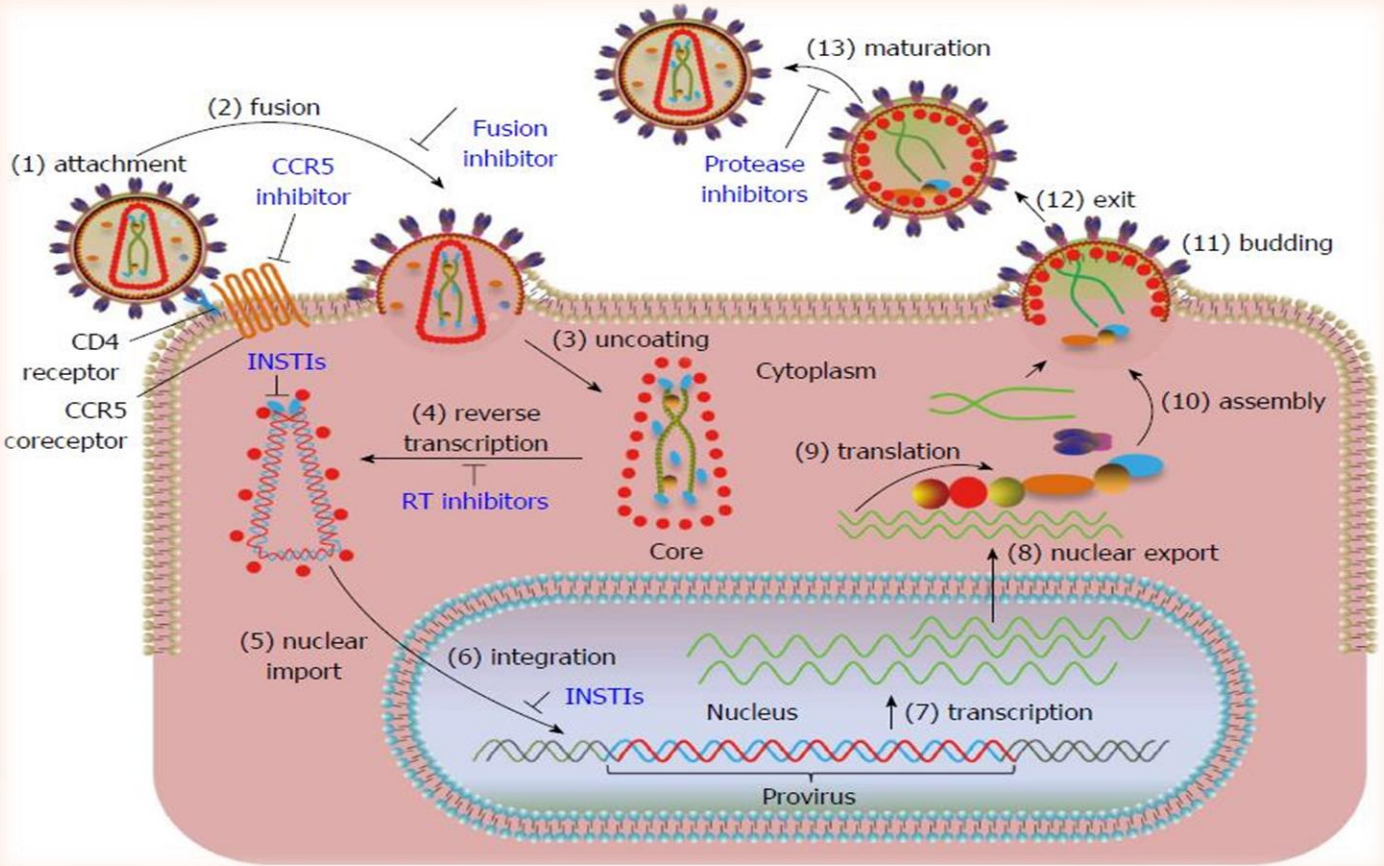
3. An interesting application of **lentivirus-based gene therapy** was the **induction of long-term tolerance of 1,3 galactosyltransferase deficient heart grafts**.

Bone marrow from gal knockout mice was transduced with a lentivirus vector expressing the porcine 1,3 galactosyltransferase and transplanted into lethally irradiated gal knockout mice.

Hearts from wildtype gal donors were permanently accepted and demonstrated that lentivirus can establish stable chimerism and induce long-term heart graft tolerance.

Overview of human immunodeficiency virus type 1 replication cycle

Grandgenett DP, Pandey KK, Bera S, Aihara H. Multifunctional facets of retrovirus integrase. *World J Biol Chem* 2015; 6(3): 83-94 [PMID: 26322168 DOI: 10.4331/wjbc.v6.i3.83]



Virus	Primary applications	Vector design	Capacity	Transduction efficiency	Transgene persistence	Patient Safety	Production scalability
Adenovirus	Vaccination, Oncolytic therapy	First generation adenovirus	5 kb	++++	+	++	++++
		Second generation adenovirus	8 kb	++++	+	++	++
		Third generation adenovirus	36 kb	++++	++	+++	++
AAV	In vivo treatment of hereditary disorders	ssAAV	5 kb	++	++	++++	+++
		scAAV	2.5 kb	+++	+++	++++	+++
		Split genome AAV	15 kb	+	++	++++	+++
Lentivirus	Ex vivo treatment of hereditary disorders, cell therapy	First generation lentivirus	8.5 kb	+++	++++	+	++
		Second generation lentivirus	8.5 kb	+++	++++	++	+
		Third generation lentivirus	8.5 kb	+++	++++	+++	+
		SIN lentivirus	8.5 kb	+++	++++	+++	+
		Integrase deficient lentivirus	8.5 kb	+++	++	++++	+

▪ **Reoviruses** have become potentially interesting cancer therapy vectors because of their preferential infection and killing of cells with an active Ras signalling pathway.

- The oncolytic nature of reoviruses renders them directly applicable to cancer treatment without the presence of any toxic or therapeutic foreign gene.

Eg:

- Reovirus killed 20 out of 24 established cancer cell lines and all 9 tested glioma biopsies from patients.
- *In vivo, complete tumor regression was* observed in mice with subcutaneous and intracerebral human glioma xenografts after injection of live reovirus.
- Studies in breast tumor-derived cell lines and biopsies from cancer patients demonstrated cell killing by reoviruses.
- Likewise, the cytopathic effect of reoviruses was showed in several human colon and ovarian cancer cell lines, but not in normal colon and ovarian cell lines.

- **SV40**

The high-titer SV40 virus production, the broad host range and the relatively weak immune response against SV40 have made them potentially attractive as gene therapy vectors.

- One drawback of SV40 is the limited packaging capacity of foreign DNA.
- The main applications of SV40 gene transfer have been on **hematopoietic progenitor cells** such as **CD34+ cells** and **liver cells**.

SV40 vectors have also been studied in relation to hepatitis B (HBV) infection.

Eg: The SV40 transduction rate of human hepatocytes was significantly higher in the HBV positive cell lines HepG2.2.2.15 and DLC4-A10II than in control HepG2 cells based on luciferase expression.

- **Vaccinia viruses** belong to the family of poxviruses, which are large dsDNA viruses that in contrast to other DNA viruses replicate in the cytoplasm. Both non-replicating and replication-competent vaccinia virus expression vectors have been engineered (**mutant vaccinia vector**).

- **Eg:** Deletions in the thymidine kinase (TK) gene and the vaccinia growth factor (VGF) gene resulted in a **mutant vaccinia vector**, which showed a high replication rate in tumor cells, but not in normal cells.

Systemic delivery of the mutant vaccinia vector in mice with subcutaneous tumors was safe and the treatment was successful.

- Recombinant vaccinia virus vectors have also been used in cytokine immunotherapy.

- **Baculoviruses,** the viruses specific for invertebrates, are not competitors of retroviruses in gene therapy.
  Baculovirus vectors have generally been associated with high-level recombinant protein expression in insect cells.
- They have been used for more than 30 years for transduction of insect cells for expression of recombinant proteins.
- Pseudotyping enables the transduction of mammalian cells (BacMam vectors).
- Vectors derived from **alfaviruses (ssRNA viruses)** are also used in cancer gene therapy and Immunotherapy.

Gene Therapy Applications of Viral Vectors

Virus	Virus Stock Preparations				Total time
AAV	Co-transfection of plasmid DNA	Virus production in HEK293 cells	Harvest of virus stock	Purification, concentration	5-6 days
Adenovirus	Co-transfection of plasmid DNA	Virus production in HEK, PerC6 cells	Harvest of virus stock	Purification, concentration	10 days
Alphavirus	In vitro transcription of rec & helper RNA	Electroporation of RNA into BHK cells	Harvest of virus stock	Purification, only for GMP	2-3 days
Baculovirus	Co-transfection of plasmid DNA	Virus production in insect cells	Harvest of virus stock	Purification, concentration	14 days
Herpesvirus	Co-transfection of amplicon & helper DNA	Virus production in Vero cells	Harvest of virus stock	Purification, ultracentrifugation	7 days
Lentivirus	Co-transfection of plasmid DNA	Virus production in HEK293 cells	Harvest of virus stock	Purification, concentration	7 days
Poxvirus	Co-transfection of plasmid DNA	Virus production in cell lines	Harvest of virus stock	Purification, concentration	7 days
Reovirus	Infection with reovirus	Virus production in L929 cells	Harvest of virus stock	Purification, concentration	3-4 days
Retrovirus	Co-transfection of plasmid DNA	Virus production in packaging cells	Harvest of virus stock	Purification, concentration	10 days
SV40	Co-transfection of plasmid DNA	Virus production in COS cells	Harvest of virus stock	Purification, ultracentrifugation	7 days

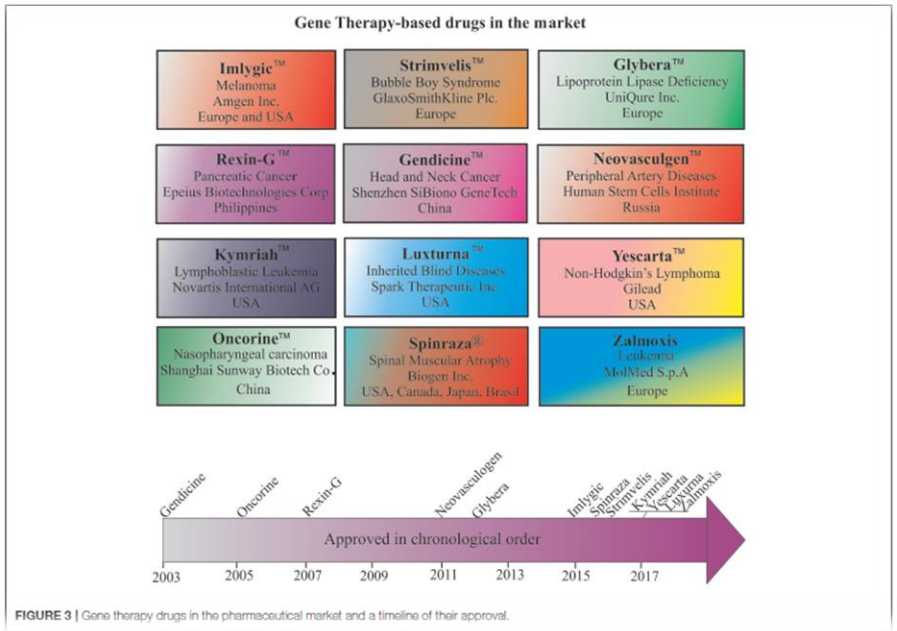
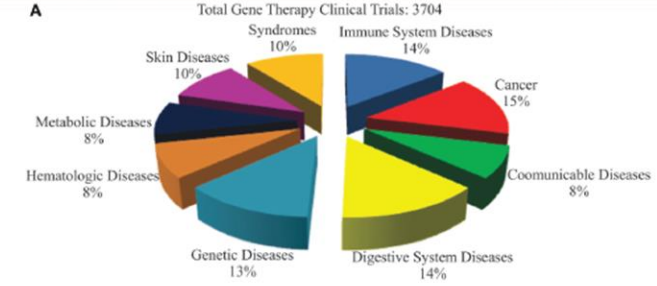
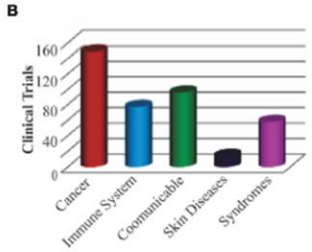


FIGURE 3 | Gene therapy drugs in the pharmaceutical market and a timeline of their approval.





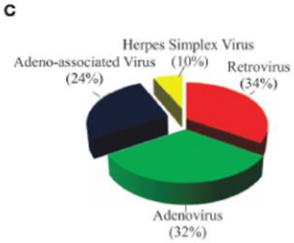


FIGURE 4 | Recent trends in gene therapy research and clinical trials. **(A)** Different diseases being treated by gene therapy in clinical trials. The clinical studies database was searched for the total number of gene therapies conducted in the world to treat different diseases to date. The main focus of the clinical trials was found to be treating cancer, immune, digestive, and genetic diseases. **(B)** Clinical trials actively recruiting patients for testing gene therapy-mediated medicines in curing diseases. This includes both viral and non-viral vector-mediated gene therapies. A relatively large number of clinical trials are recruiting cancer patients for testing different gene therapy-based medicines. **(C)** Different recombinant viral vectors being tested in gene therapy-based treatments.

Chapter 5

Retroviral Vectors in Gene Therapy

Miroslava Matuskova and Erika Durinikova

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61844

Technology in Cancer Research & Treatment
ISSN 1533-0346
Volume 3, Number 5, October (2004)

Gene Therapy Applications of Viral Vectors

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Viral vectors have frequently been applied in gene therapy with the final goal of treating various diseases in the areas of neurology, neurodegeneration, metabolic disease, and cancer. Vectors have been engineered based on AAV, adenoviruses, alphaviruses, herpes simplex viruses, lentiviruses, and retroviruses. Some vectors are suitable for short-term episomal transgene expression, whereas others are integrated into the host cell genome to provide long-term expression. Additionally, hybrid vectors with favorable features from different viruses have been developed. Therapeutic genes of choice have typically been toxic genes such as thymidine kinase, pro-apoptotic genes like Bax, and immunostimulatory genes (for instance, interleukin-12). A large number of animal studies have demonstrated proof of con-

frontiers in Oncology

REVIEW
published: 24 April 2019
doi: 10.3389/fonc.2019.00297

Gene Therapy Leaves a Vicious Cycle

*Reena Goswami[1], Gayatri Subramanian[2], Liliya Silayeva[1], Isabelle Newkirk[1], Deborah Doctor[1], Karan Chawla[2], Saurabh Chattopadhyay[2], Dhyan Chandra[3], Nageswararao Chilukuri[1] and Venkaiah Betapudi[1,4]**

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The human genetic code encrypted in thousands of genes holds the secret for synthesis of proteins that drive all biological processes necessary for normal life and death. Though the genetic ciphering remains unchanged through generations, some genes get disrupted, deleted and or mutated, manifesting diseases, and or disorders. Current treatment options—chemotherapy, protein therapy, radiotherapy, and surgery available for no more than 500 diseases—neither cure nor prevent genetic errors but often cause many side effects. However, gene therapy, colloquially called "living drug," provides a one-time treatment option by rewriting or fixing errors in the natural genetic ciphering. Since gene therapy is predominantly a viral vector-based medicine, it has met with a fair bit of skepticism from both the science fraternity and patients. Now, thanks to

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