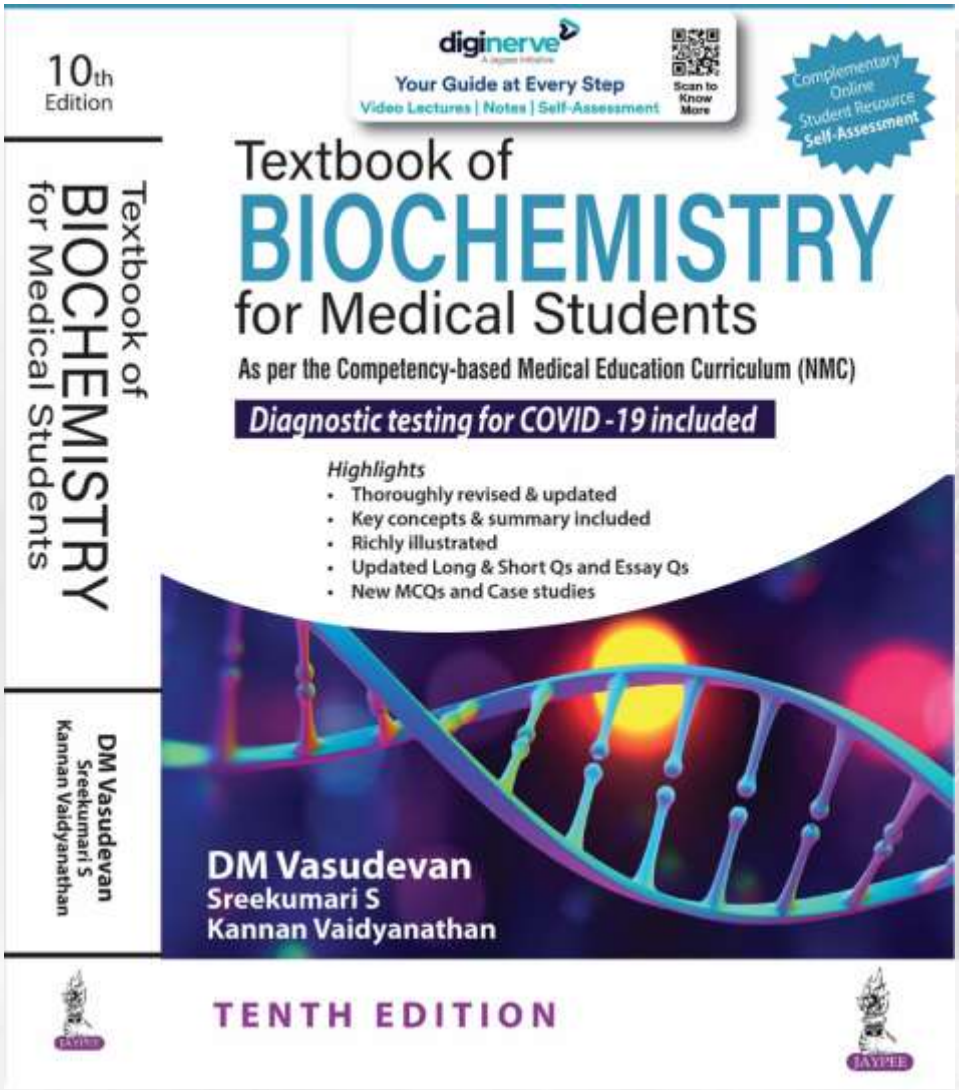


Chapter 37: Recombinant DNA Technology, Gene Therapy

Textbook of
BIOCHEMISTRY
for Medical Students

By DM Vasudevan, *et al.*

TENTH EDITION

Recombinant DNA Technology

Biotechnology may be defined as "the method by which a living organism or its parts are used to incorporate a particular character to another living organism".

"Genetic Engineering"

Applications of Recombinant Technology

1. Quantitative Preparation of Bio-molecules

If molecules are isolated from higher organisms, the availability will be greatly limited. For example, to get 1 unit of growth hormone, more than 1000 pituitaries from cadavers are required. By means of recombinant technology, large scale availability is now assured.

2. Risk of Contamination is Eliminated

It is now possible to produce a biological substance without any contamination. Hepatitis, caused by the hepatitis B virus (HBV), is highly contagious. Originally the virus was isolated from pooled blood.

Applications of Recombinant Technology

- Antenatal diagnosis of genetic diseases. Single gene defects (e.g. cystic fibrosis, phenylketonuria, etc.) could be identified by taking cell samples from the fetus.
- To identify viral particles or bacterial DNA in suspected blood and tissue samples.
- To detect activation of oncogenes in cancer.
- To pinpoint the location of a gene in a chromosome.
- Point mutations, deletions, insertions and rearrangements of DNA could be identified. Sickle cell disease is an example of point mutation. The substitution of T for A in the template strand of DNA in the beta globin gene changes the Mst II restriction site
- **Gene therapy :** Normal genes could be introduced into the patient so that genetic diseases can be cured.

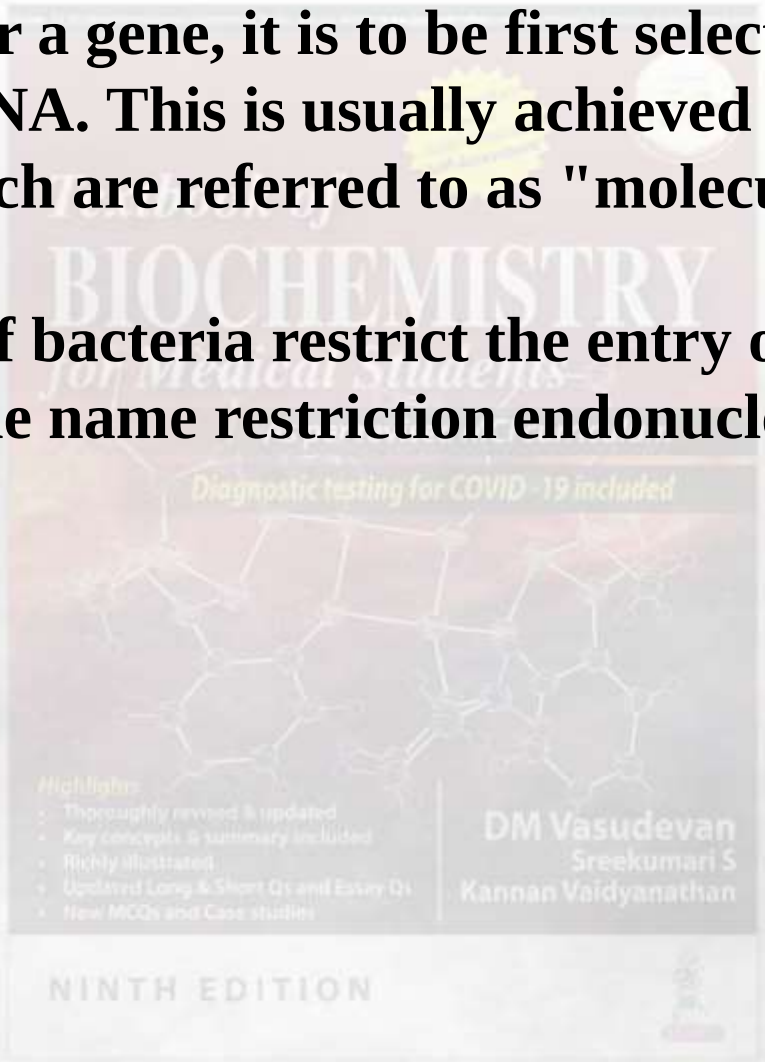
Biotechnology

- ANTIBI0TICS AND DRUG DEVELOPMENT
- RECOMBINANT HORMONES AND ENZYMES
- DROUGHT AND INSECT RESISTANT CROPS
- BIOFERTILIZERS AND BIOINSECTISIDES

Restriction Endonucleases (RE)

In order to transfer a gene, it is to be first selectively split from the parent DNA. This is usually achieved by restriction endonucleases which are referred to as "molecular scissors".

Certain enzymes of bacteria restrict the entry of phages into host bacteria. Hence, the name restriction endonucleases.



Specificity of Restriction Enzymes

(The arrows show the site of the cut by the enzyme)

Eco RI	**E Coli**	G AATT C C TTAA G
Hind III	**Haemophillus influenza**	A AGCT T T TCGA A
Taq I	**Thermus aquaticus**	T CG A A GC T
Hpa I	**Haemophillus parainfluenza**	GTT AAC CAA TTG

Restriction map of DNA from lambda bacteriophage. The numbers denote the length of restriction fragment in kbp.

Total RNA isolated, containing mRNAs and rRNAs

↓

Pass through a column with immobilized oligo-dT, when mRNA with poly-A tail are retained, as T pairs with A

↓

mRNAs eluted; these are of different sizes coding for making different proteins

↓

Fractionate mRNA species; specific insulin-mRNA is identified and separated by passing through column

↓

DNA synthesis by reverse transcriptase + dNTPs; RNA–DNA hybrid is produced

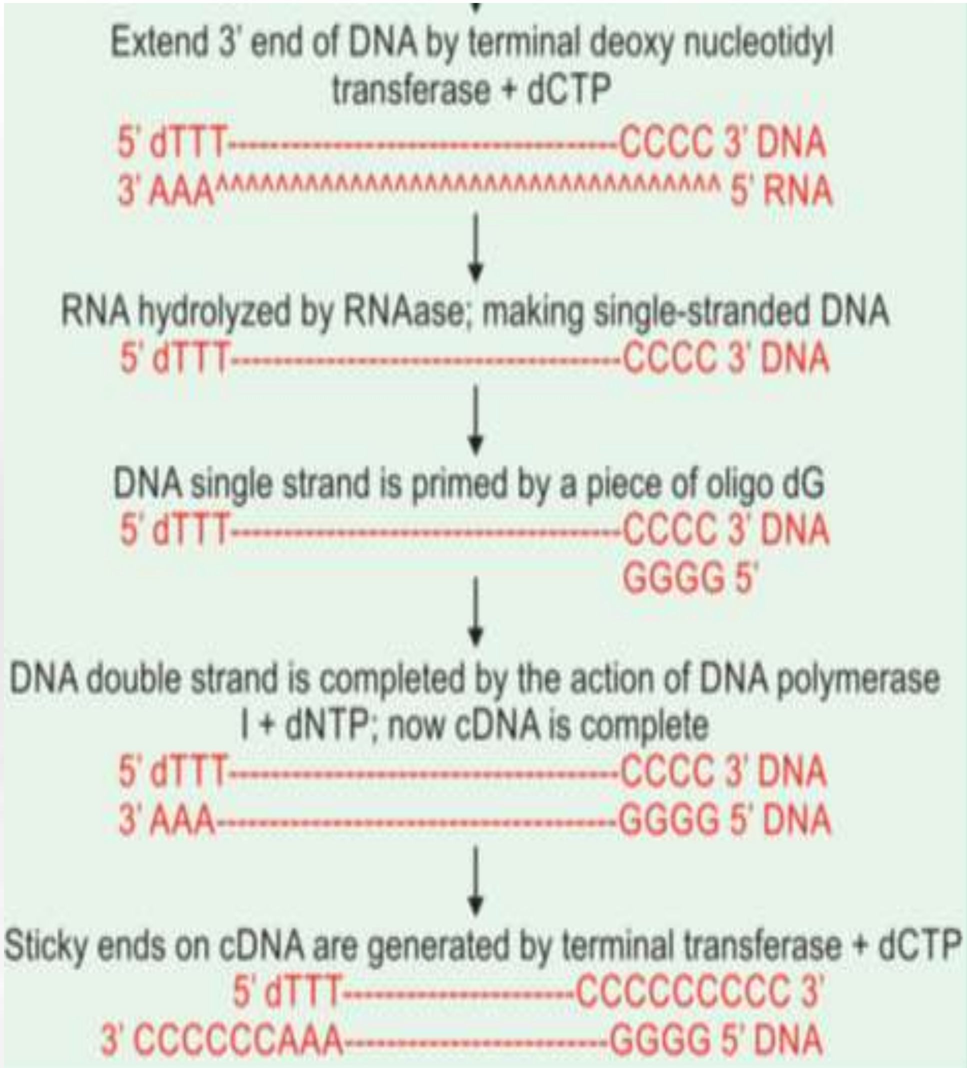
5′ dTTT------------------------------------3′ DNA
3′ AAA^^^^^^^^^^^^^^^^^^^^^^^^^^^^^5′ RNA

↓

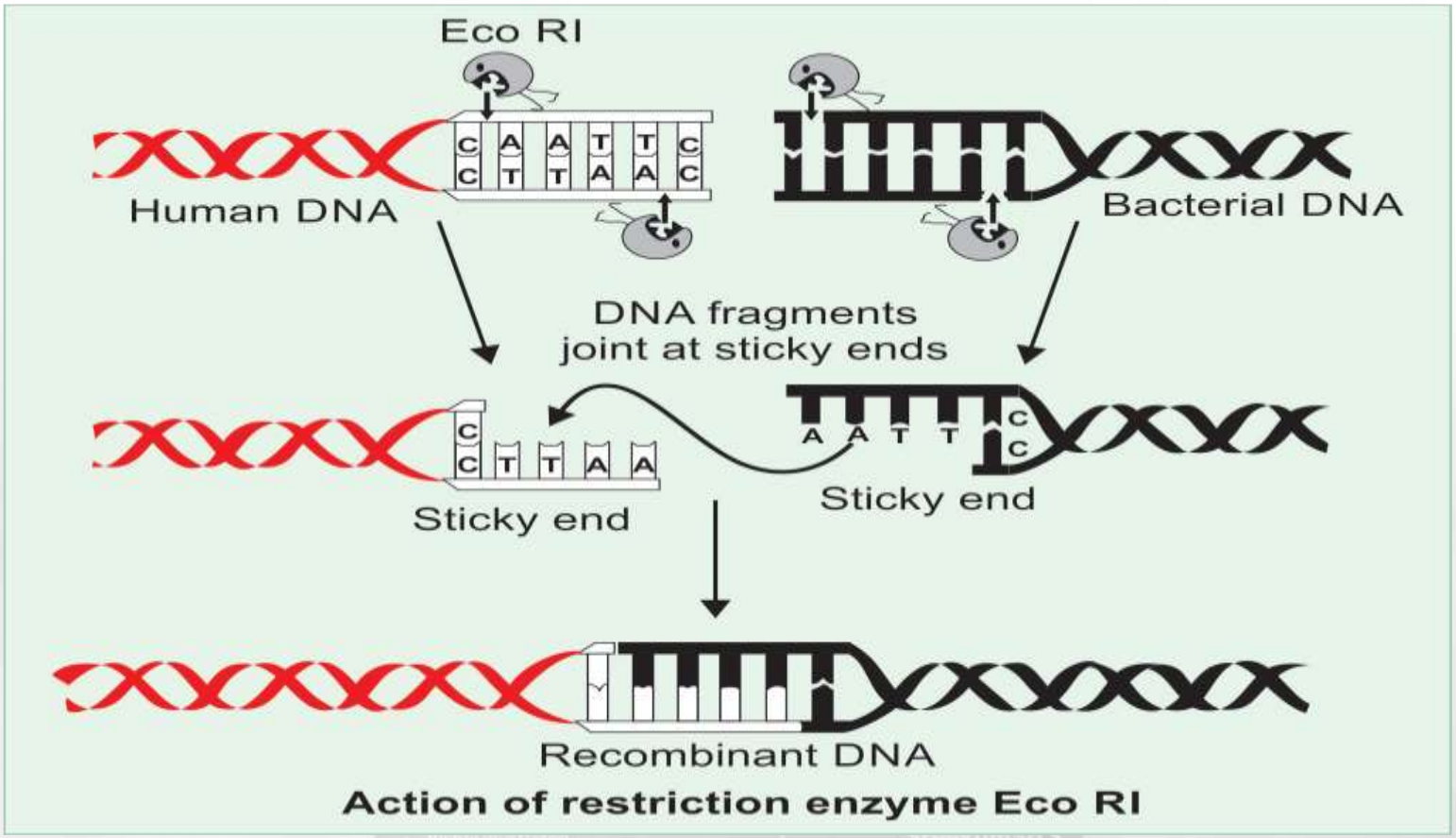
Extend 3′ end of DNA by terminal deoxy nucleotidyl transferase + dCTP

5′ dTTT------------------------------------CCCC 3′ DNA
3′ AAA^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^ 5′ RNA

Steps to produce cDNA from mRNA.

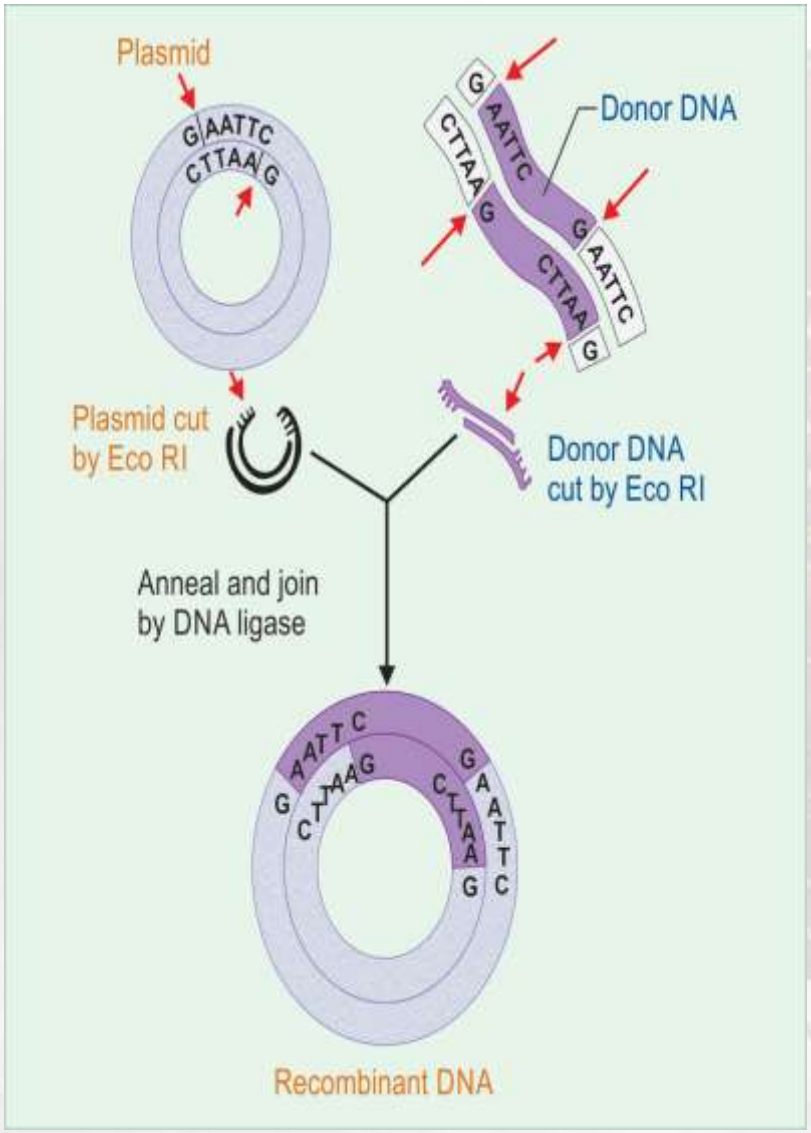


Steps to produce cDNA from mRNA, continued



Action of restriction enzyme Eco RI

EcoRI enzyme cuts the bonds marked with arrow.
This results in the sticky ends.



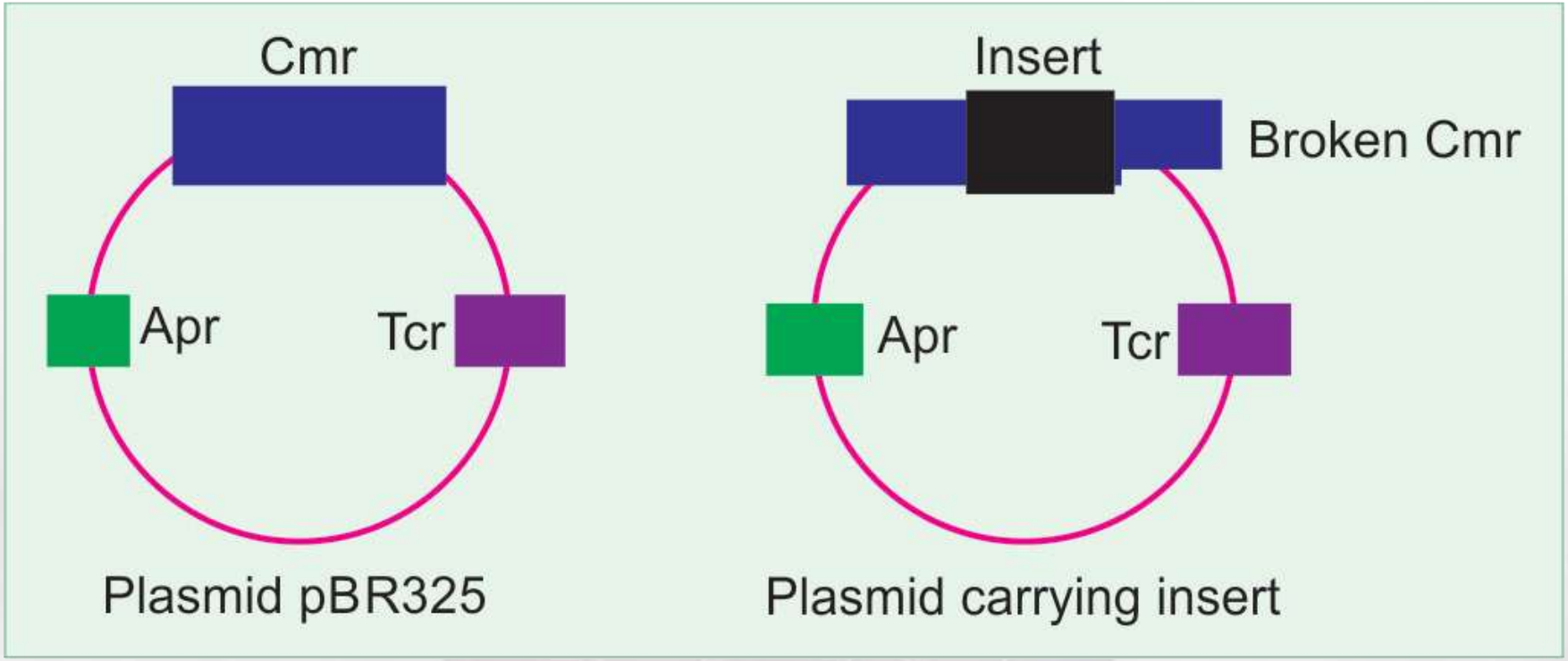
Production of chimeric DNA molecule by using ECoRI restriction endonuclease. Red arrows show the site of cut by ECoRI.

Transfection of Vector into the Host

Host E.coli cells and plasmid vectors are incubated in hypertonic medium containing calcium for a few minutes.

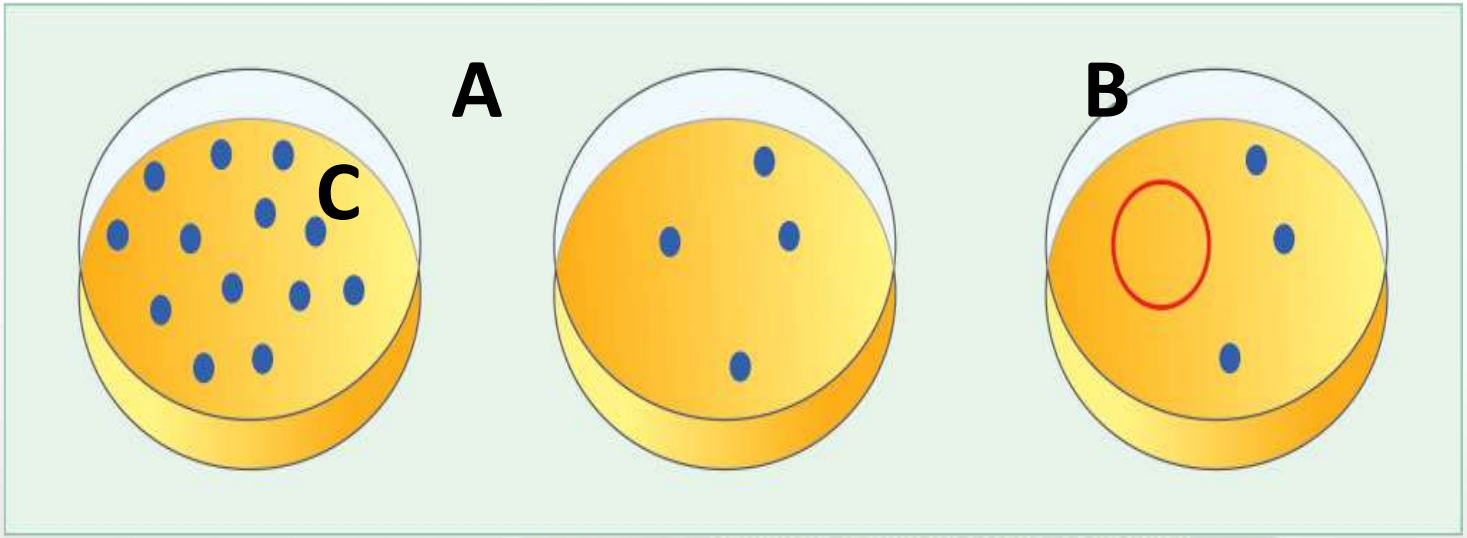
Now the host cells are allowed to grow in agar plates containing growth medium.

Only 5% of bacterial colonies contain the desired vector. Therefore, we have to select the desired colonies.



Insertional inactivation of Cmr gene of plasmid.
(Apr: ampicillin resistance gene;
Cmr: chloramphenicol resistance gene;
Tcr: tetracycline resistance gene).

Transfection of Vector into the Host



A= E.coli bacteria growing in ordinary medium having many colonies.

B = Growth in medium containing ampicillin and tetracyclin; only few colonies.

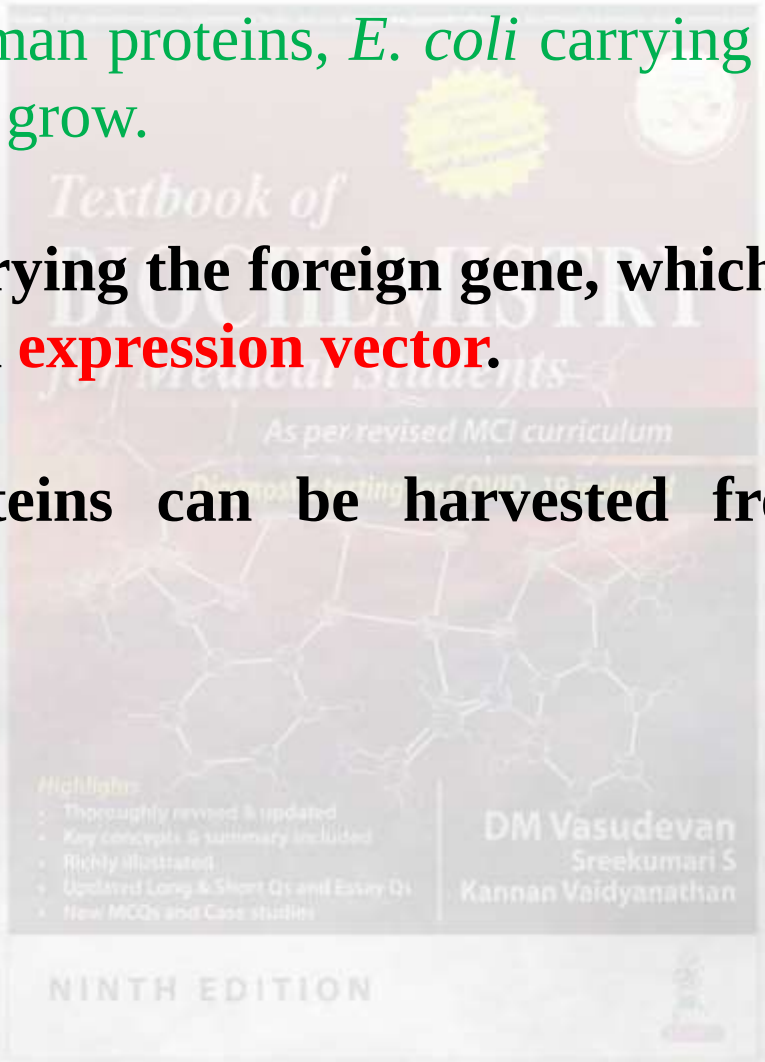
C = Replica plate with chloramphenicol where chloramphenicol sensitive colony (marked as red circle) is absent.

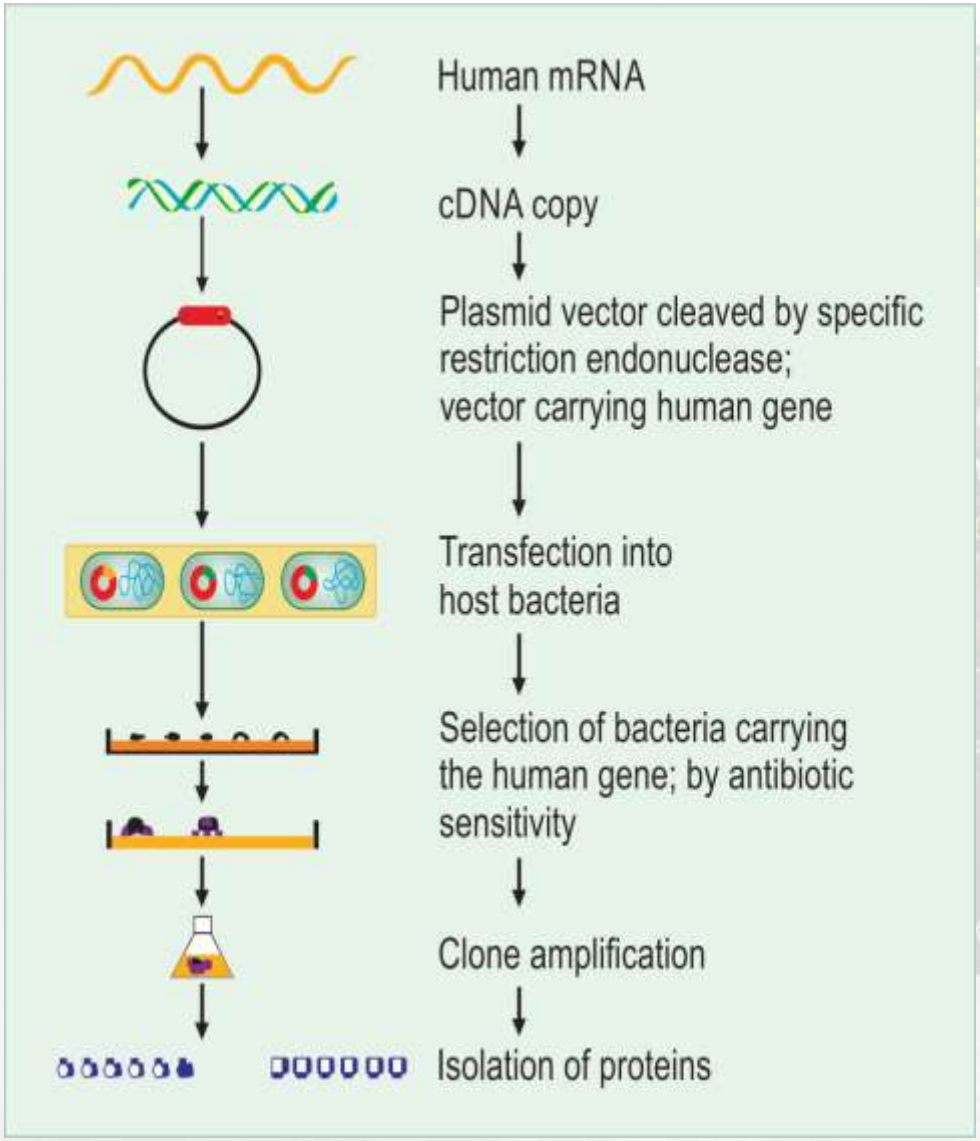
That colony in B is selected for further amplification.

To produce the human proteins, *E. coli* carrying the vector with the insert is allowed to grow.

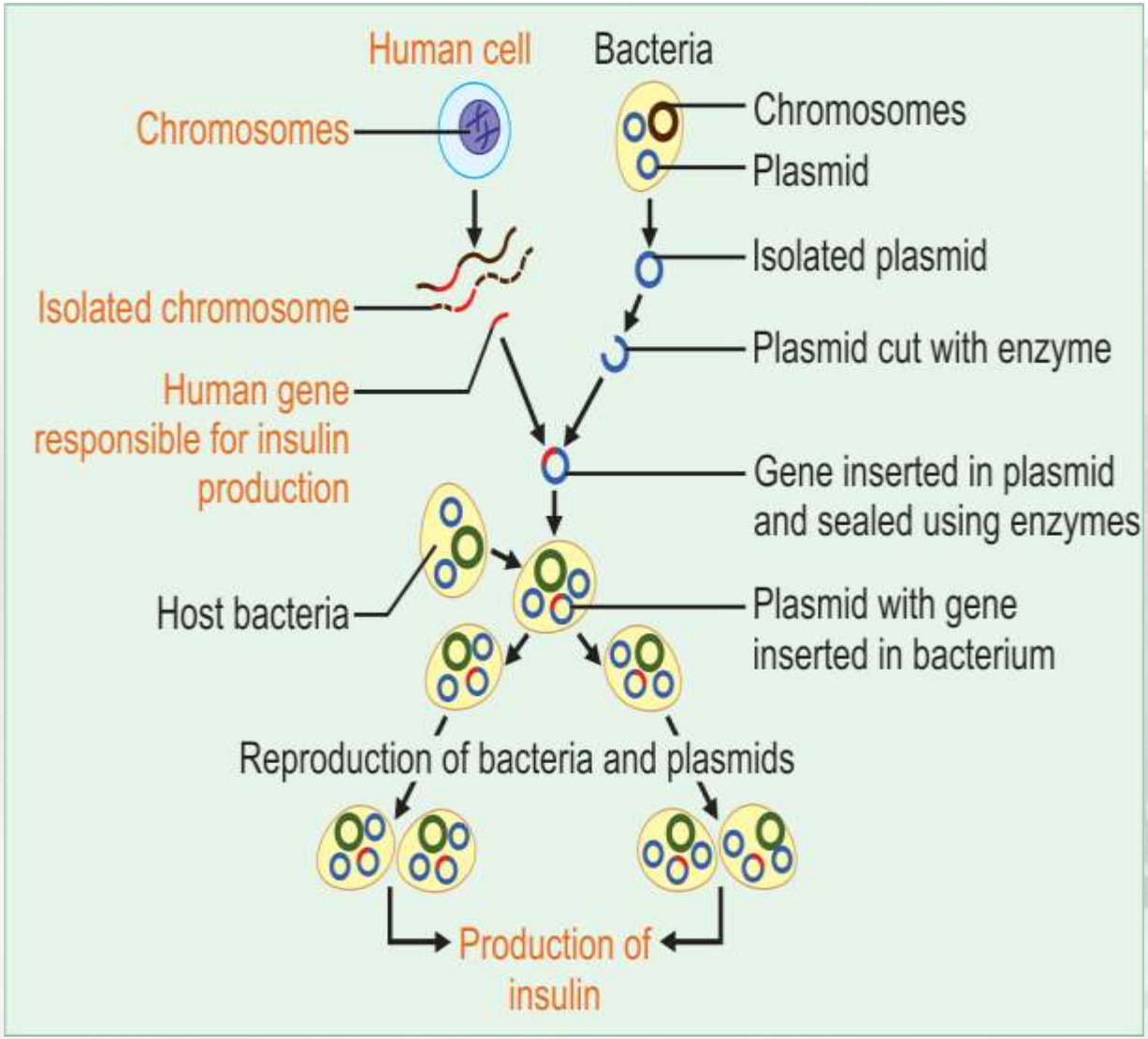
Such a vector carrying the foreign gene, which is translated into a protein, is called expression vector.

The human proteins can be harvested from the bacterial culture.





DNA recombinant technology.



Scheme to produce recombinant human insulin.

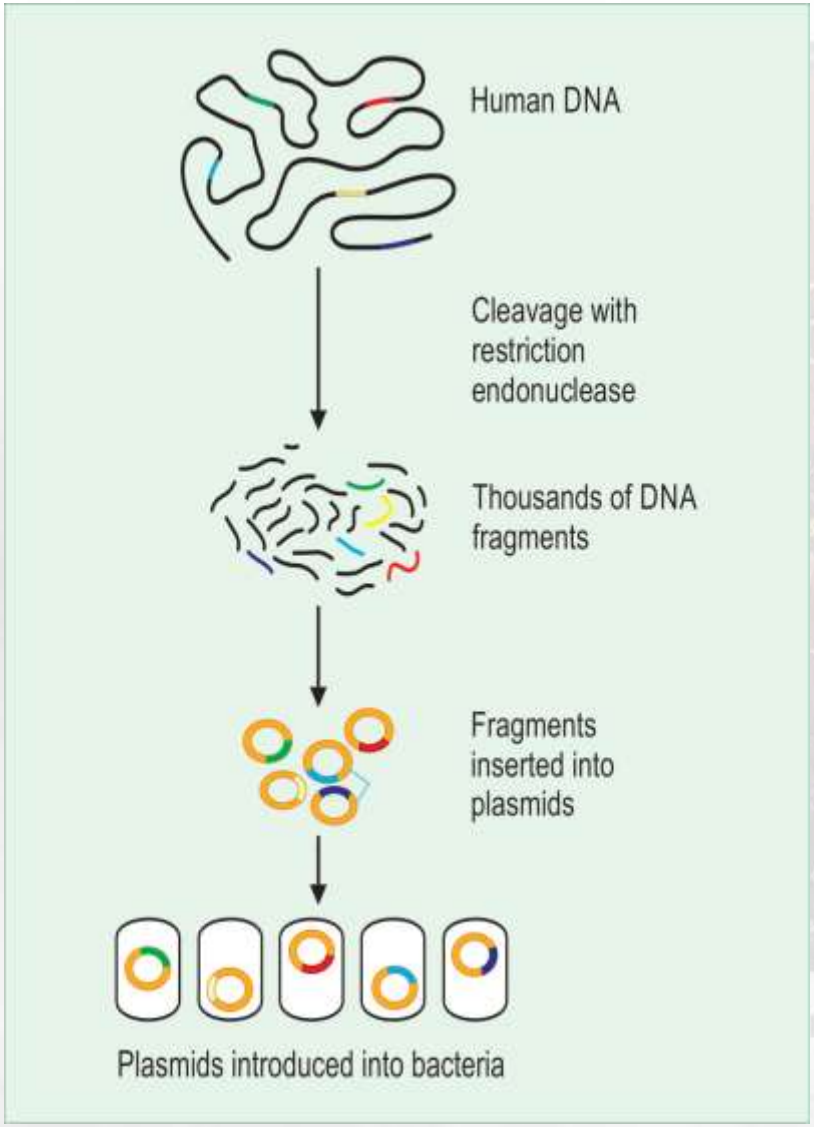
Selected list of proteins produced by recombinant DNA technology

- Recombinant human insulin to treat diabetes mellitus
- Recombinant human growth hormone (HGH)
- Other human hormones (e.g. FSH)
- Recombinant blood clotting factor VIII and other clotting factors (Factor IX) for treatment of hemophilia
- Recombinant vaccines, such as hepatitis B vaccine, HPV vaccine
- Cytokines and growth factors (interferon, interleukins)
- Monoclonal antibodies and other related products (rituximab, trastuzumab, etc.)
- Recombinant enzymes (acid alpha-glucosidase, alpha-L-iduronidase)
- Recombinant HIV protein for HIV ELISA testing
- Tissue plasminogen activator, used to treat strokes

Antibiotic Resistance

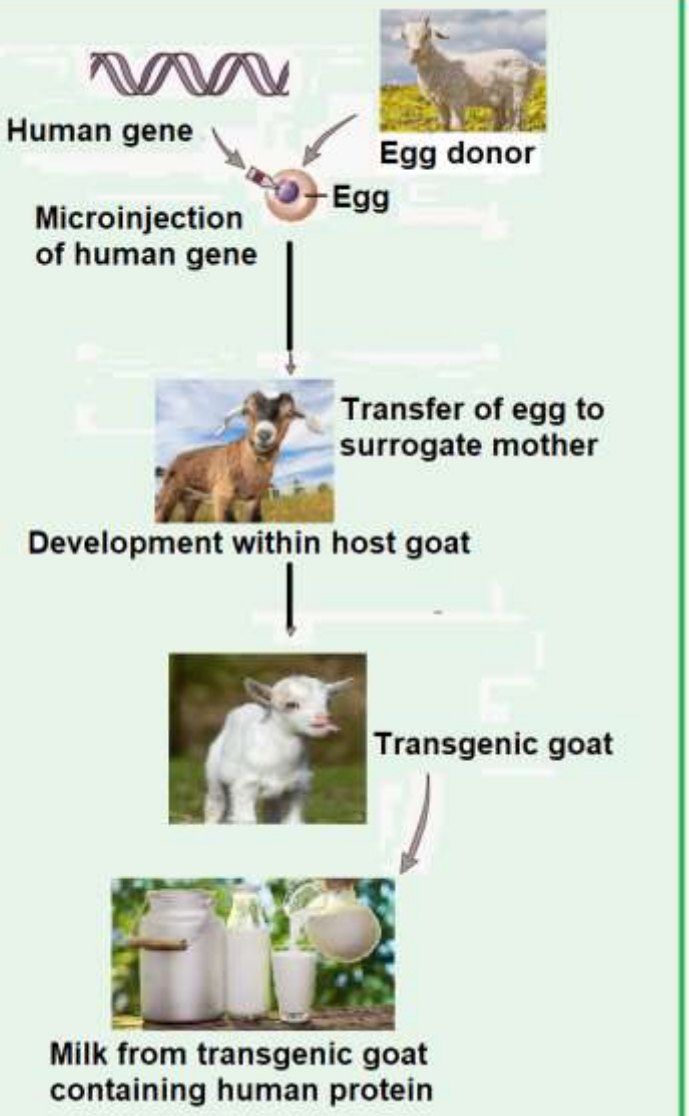
Antibiotic resistance occurs when an antibiotic has lost its ability to effectively control or kill bacterial growth. When an antibiotic is used, susceptible bacteria are killed, resulting in a selective advantage for the survival of resistant strains of bacteria. Bacteria may become resistant in two ways: 1) by a genetic mutation or 2) by acquiring resistance from another bacterium. Bacteria can acquire antibiotic resistance genes from other bacteria through plasmid exchange.

Multiple antibiotic resistances represent a serious clinical problem with regard to bacterial infections in humans. The antibiotic resistance spreads through bacterial populations both “vertically," and "horizontally". The discovery of a new antibiotic will take decades; but resistant bacteria to the new antibiotic will emerge within years. Thus bacteria have the upper hand in the race with human beings.



Production of genomic library.

Transgenic animals



Cloned genes may be inserted into organisms, generating transgenic organisms. These animals have a foreign gene deliberately inserted into their genome; usually foreign DNA is microinjected into the pronuclei of a fertilized egg which is subsequently implanted into the oviduct of a surrogate mother. Thus human proteins can be harvested in the milk of transgenic animals.

Proteins produced in milk of transgenic animals

Human protein	Animal species
Tissue plasminogen activator (tPA)	Goat
Interleukin-2	Rabbit
Factor VIII	Pig
Fibrinogen	Sheep
Insulin like growth factor	Rabbit
Growth hormone	Rabbit

Human Genome Project

- International effort started in 1990

  Goals

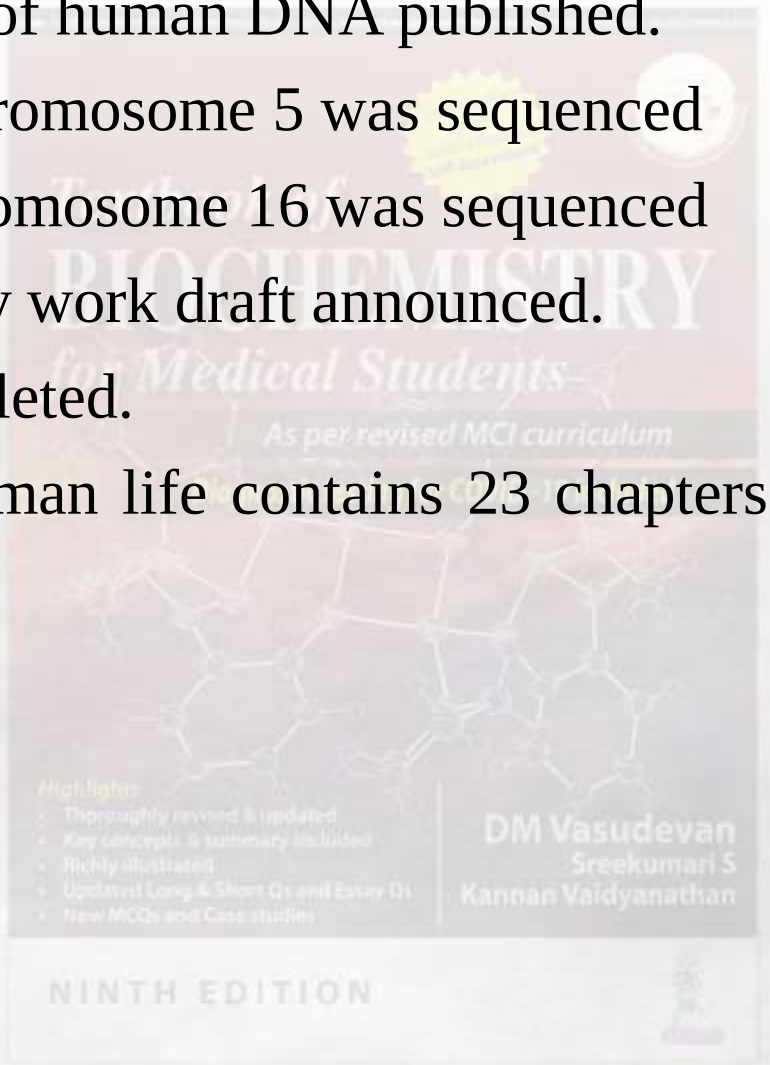
- Sequencing the entire genome

  One set of human DNA contains 3 billion base pairs and about 10,000genes.

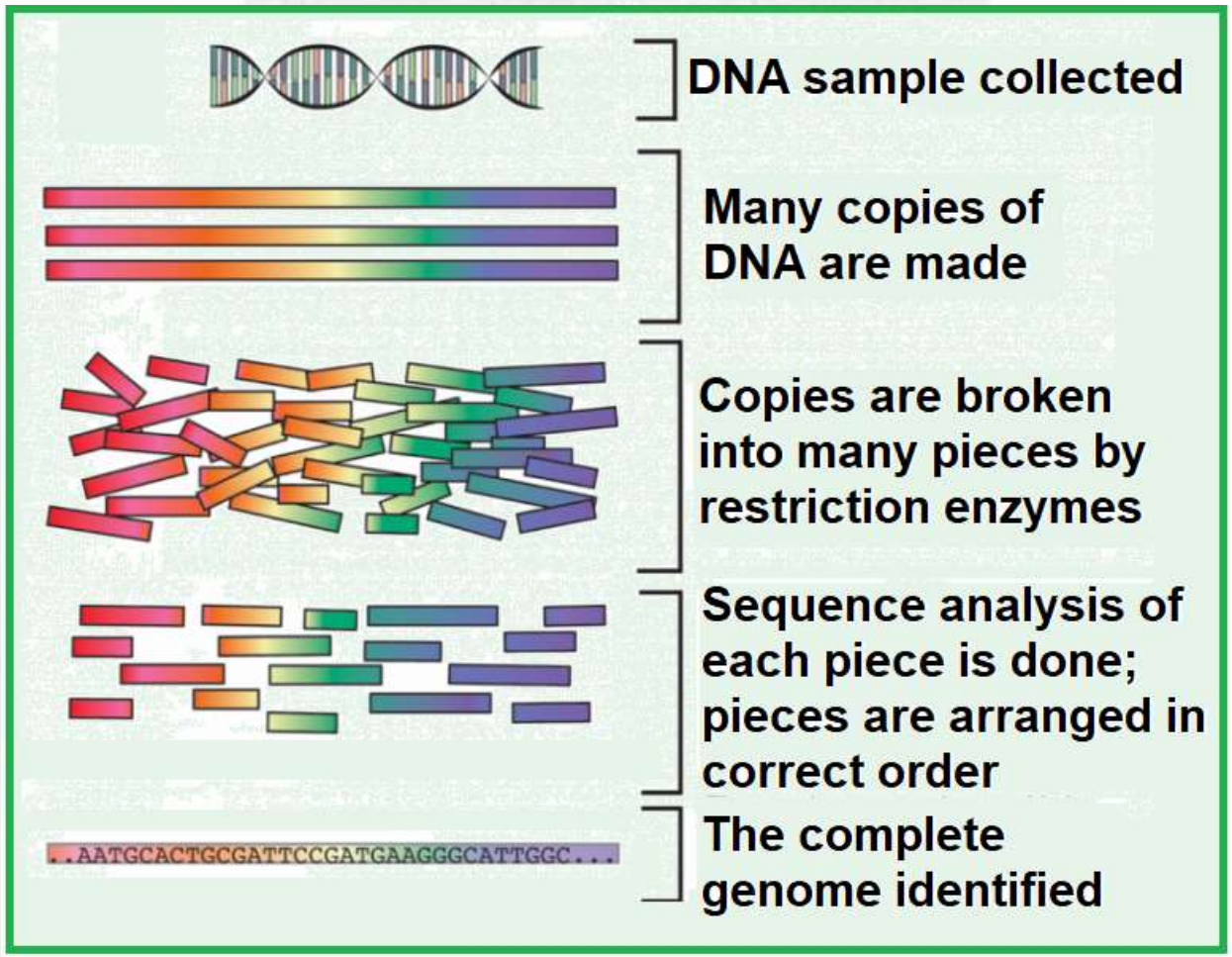
- US department of Energy and US National Institute of Health started the project in 1990.
- James Watson headed the project first, later Francis Collins succeeded him.
- The project included scientists from 16 countries world wide.
- In 1997, Craig Ventor of Celera Genomics had independently embarked on the project and hastened the completion of the project by 2002

Human Genome Project

- 1995- Gene map of human DNA published.
- 1998 – human chromosome 5 was sequenced
- 1999- human chromosome 16 was sequenced
- 2000- Preliminary work draft announced.
- 2002- HGP completed.
- The book of Human life contains 23 chapters which represent 23 chromosomes.



Human Genome Project



Whole genome sequencing; Shotgun technique.

Human Genome Project

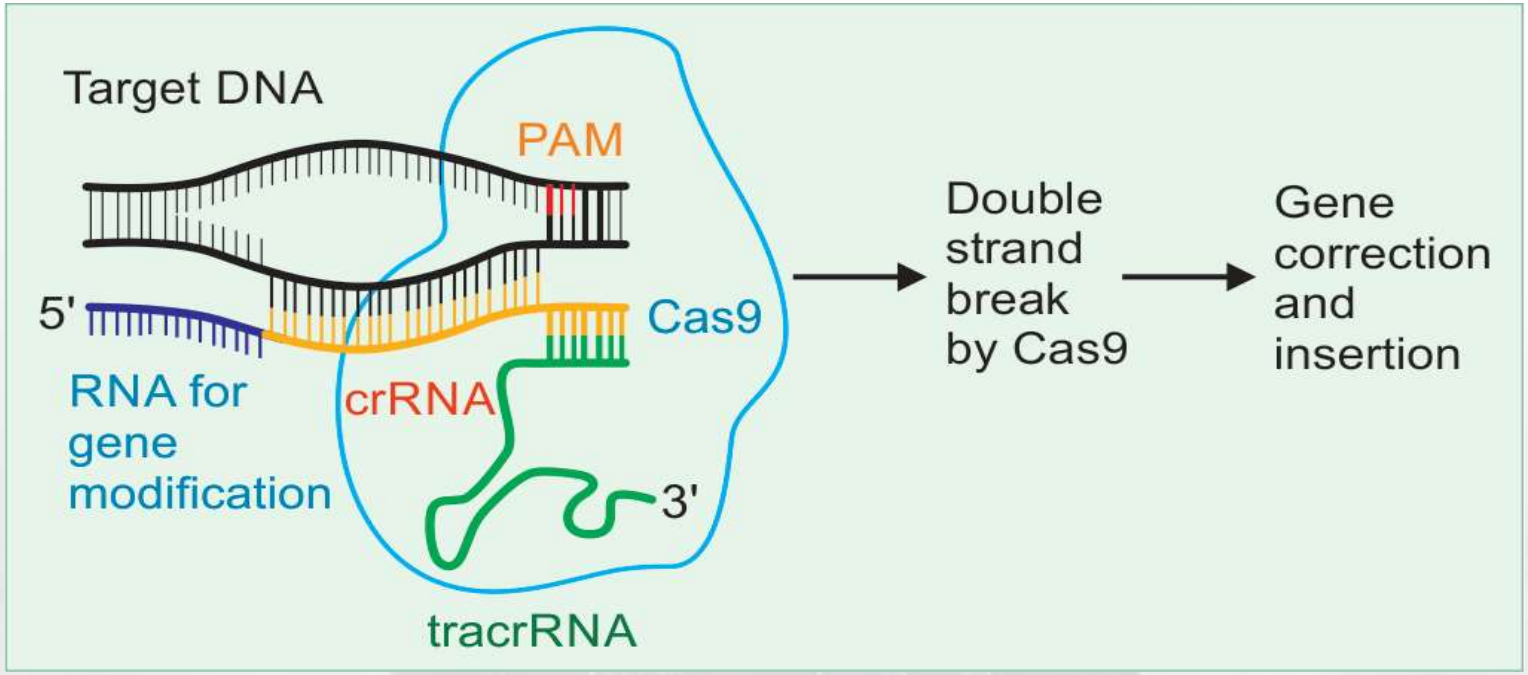
It is now possible to isolate any human gene of interest. Many previously unknown genes have been identified.

Pharmacogenomics is emerged from the genome project; it is the use of genetic information toward the development of new drugs and their targets of action.

In 2003, the National Human Genome Research Institute initiated the **ENCODE** (Encyclopedia of DNA Elements) Project, to identify all the functional elements of the genome.

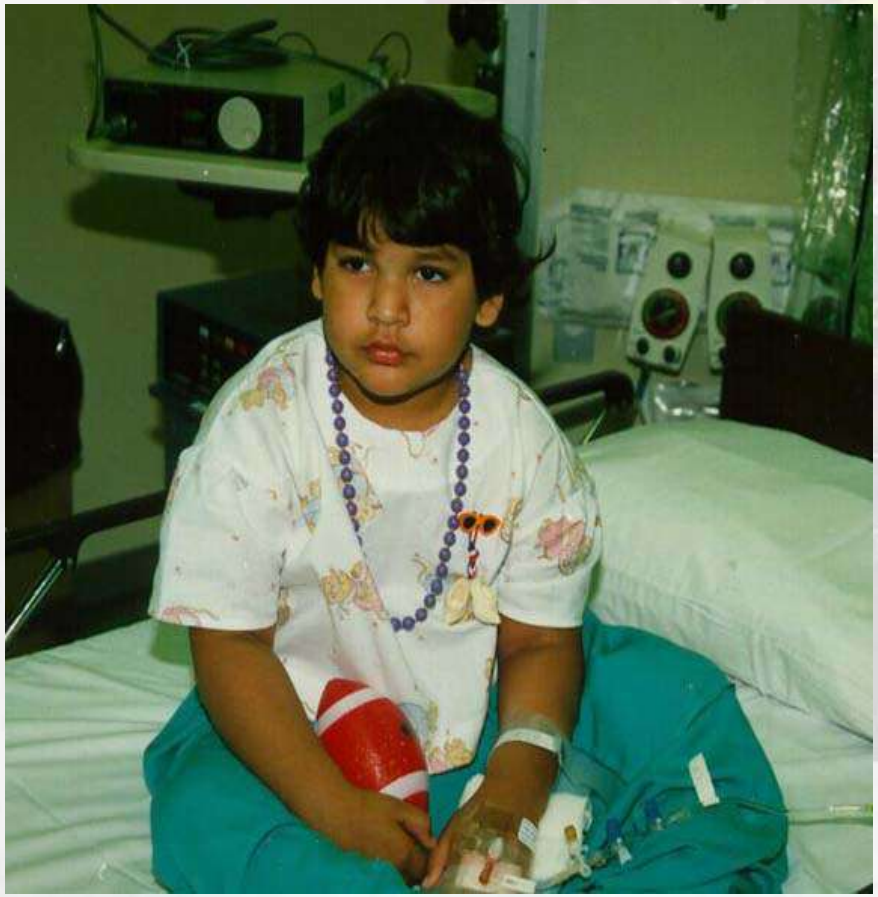
CRISPR-Cas9

The word CRISPR (pronounced as crisper) stands for "clusters of regularly interspaced short palindromic repeats". Cas stands for "CRISPR-associated system". CRISPR and cas proteins form CRISPR/Cas systems. CRISPR is another mechanism employed by the microbes which provide acquired immunity
against viruses and plasmids. The protein Cas9 is an enzyme that acts like a pair of molecular scissors, capable of cutting DNA strands. Short DNA sequences known as PAM (protospacer adjacent motifs) serve as tags present on the target DNA sequence. In the case of bacteria, the spacers are taken from the viruses that previously attacked the organism. They serve as memories, which enables bacteria to recognize the viruses and fight off future attacks. With CRISPR, it is now possible to modify, delete, insert, activate or inactivate any gene. CRISPR can be combined with gene therapy so that the modified gene can be delivered anywhere in the body.



A duplex of crRNA and tracrRNA acts as guide RNA to introduce a specifically located gene modification upstream of the crRNA. Cas9 binds the tracrRNA and needs a DNA binding sequence called protospacer adjacent motif (PAM). After binding, Cas9 produces a DNA double strand break, which is then followed by gene modification via homologous recombination (HDR) or nonhomologous end joining (NHEJ).

A great leap in medical science has taken place on the 14th September 1990.



Event: The first gene therapy trial for an inherited disorder was initiated on

The patient:

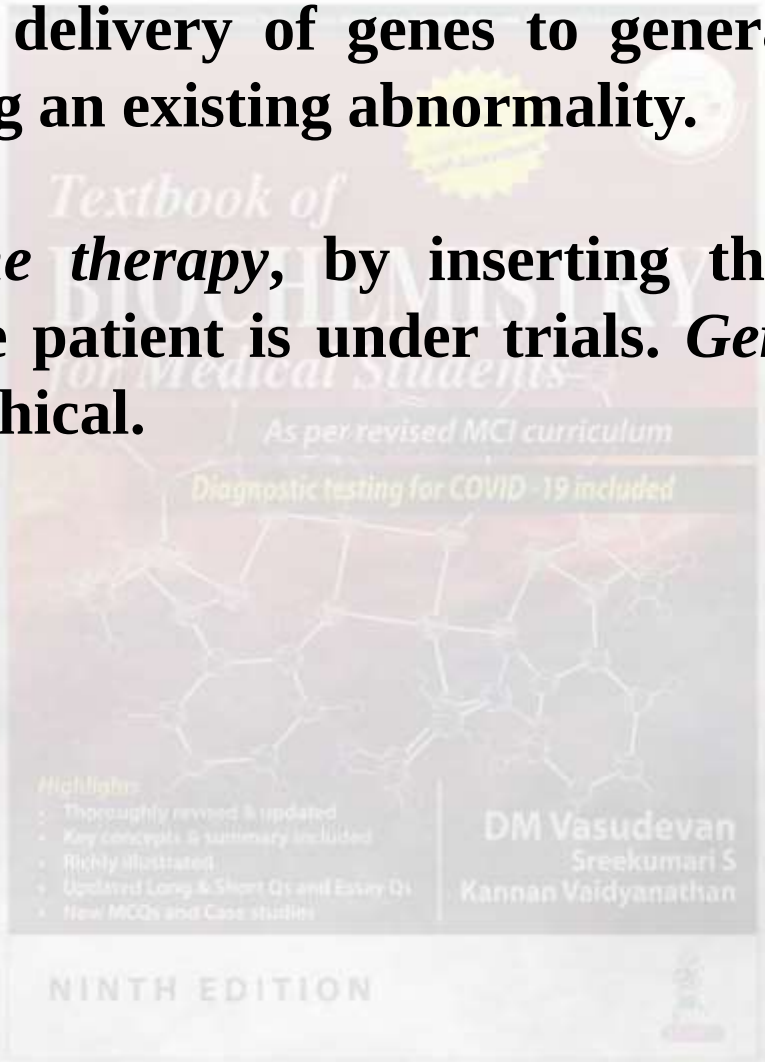
Ashanthi DeSilva, 4 year old

Disorder treated: Adenosine deaminase (ADA) deficiency.

What is Gene Therapy?

It is intracellular delivery of genes to generate a therapeutic effect by correcting an existing abnormality.

Only *somatic gene therapy*, by inserting the new gene into somatic cell of the patient is under trials. *Germ line therapy* is considered as unethical.



Summary of the Procedure

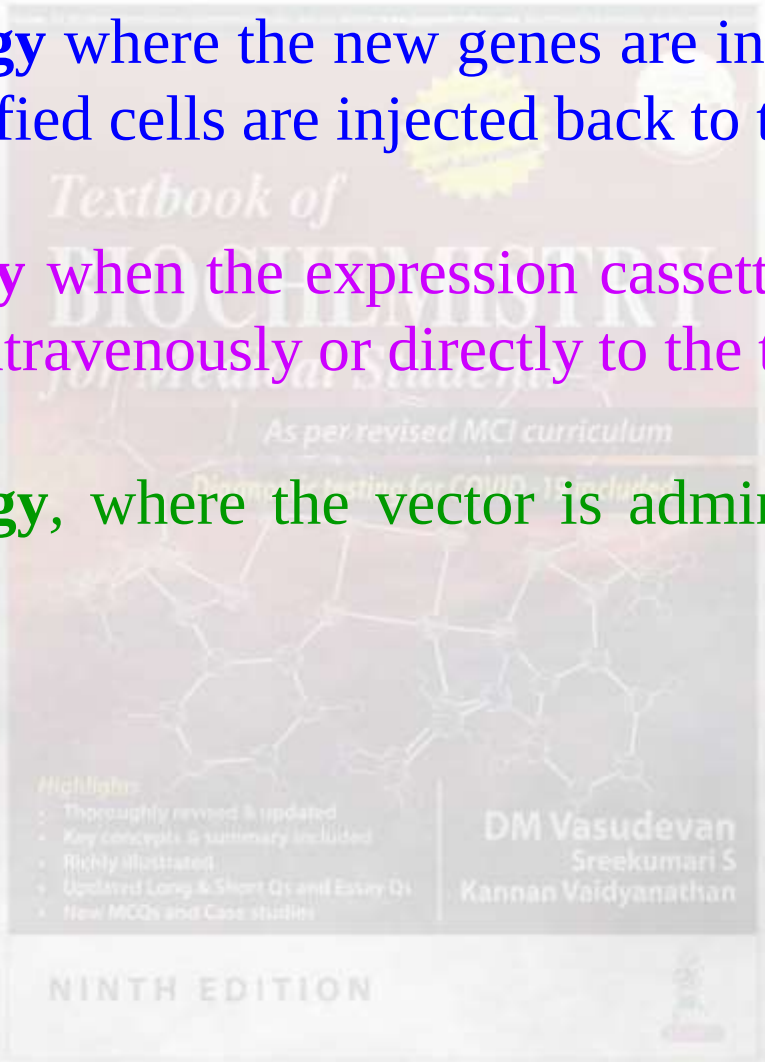
1. **Isolate the healthy gene.**
2. **Incorporate this gene on a carrier or vector as an expression cassette.**
3. **Deliver the vector to the target cells.**

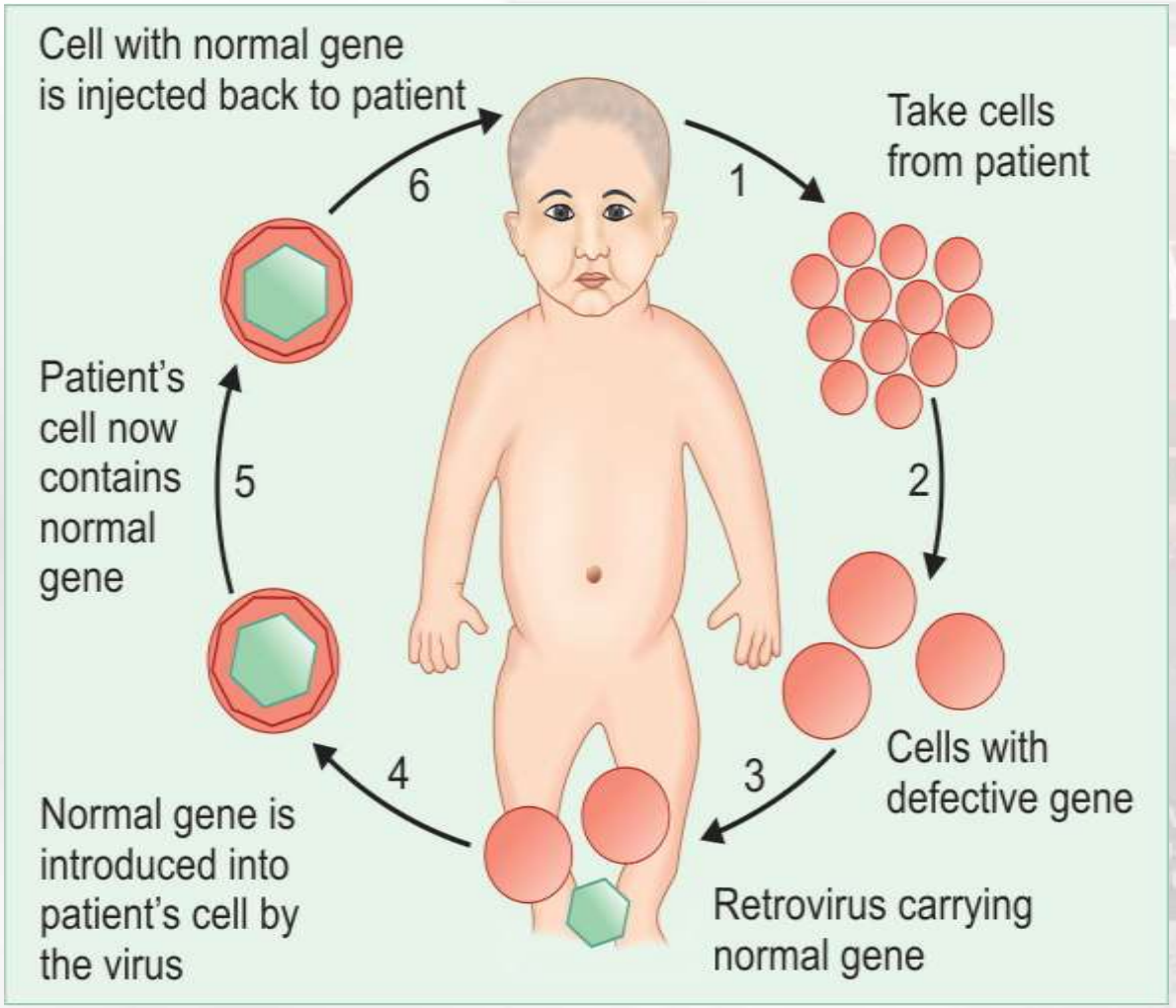
How the Genes are Introduced?

a) **Ex vivo strategy** where the new genes are infused into patient's cells; and modified cells are injected back to the patient.

b) **In situ strategy** when the expression cassette is injected to the patient either intravenously or directly to the tissue.

c) **In vivo strategy**, where the vector is administered directly to the cell.





Ex vivo gene therapy.

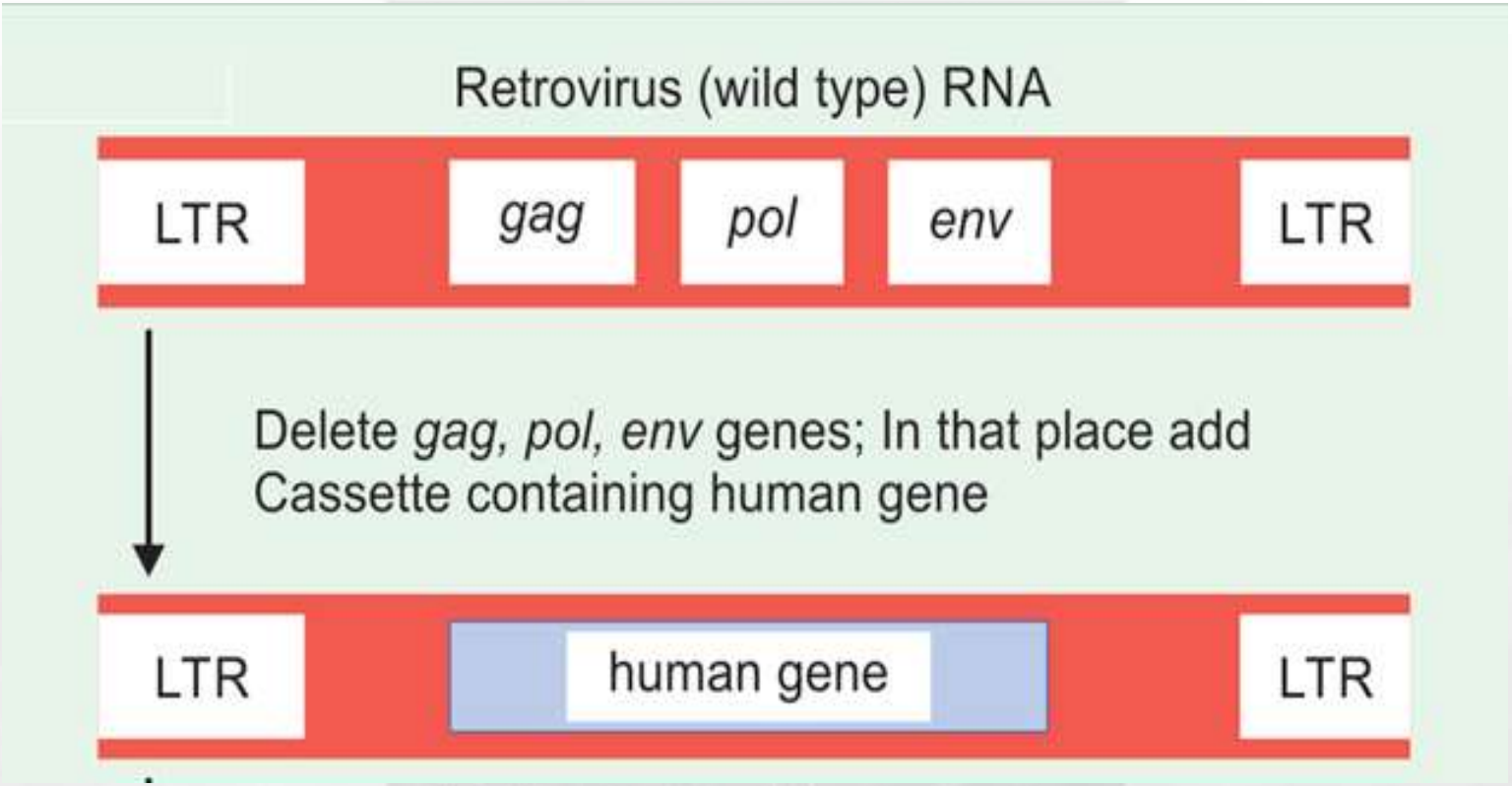
The Vectors

Retroviruses; Adenoviruses; Herpes simplex Non-virus systems : liposomes, plasmids.

Retro viruses are RNA viruses that replicate through a DNA intermediate.
Moloney Murine Leukemia Virus (MMLV) is commonly used.

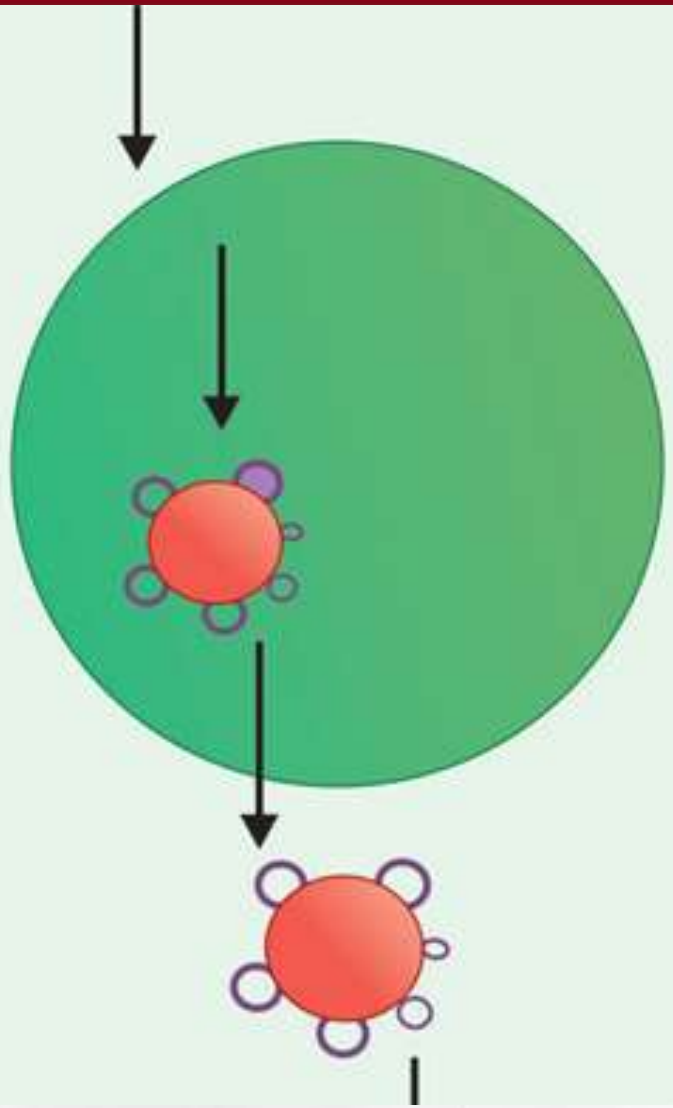
The gag, pol, env genes are deleted from the wild type retrovirus, rendering it incapable of replication inside human body.

Gene Transfer by retroviral vector



Gene transfer by retroviral vector.

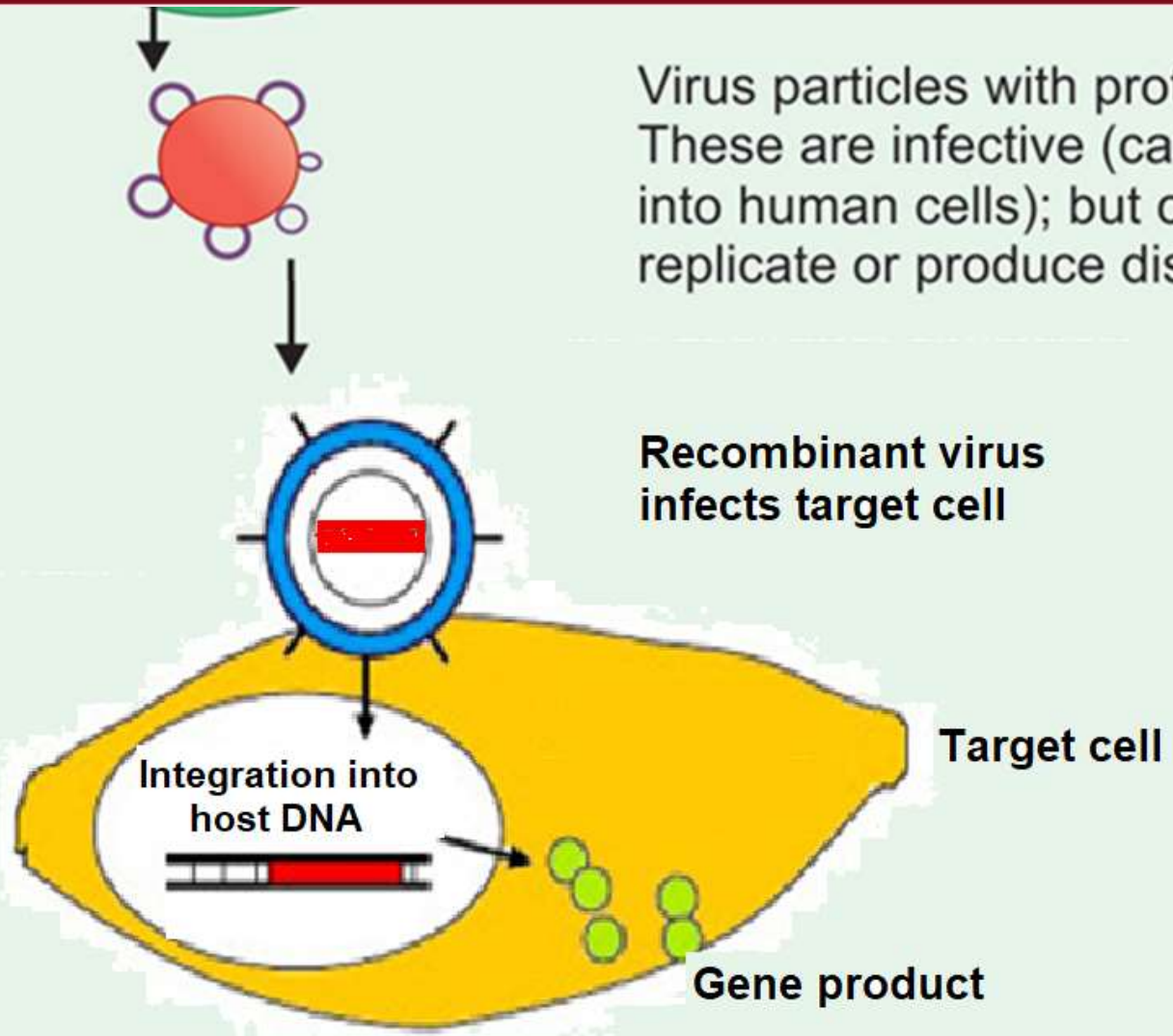
Gene Transfer by retroviral vector, continued



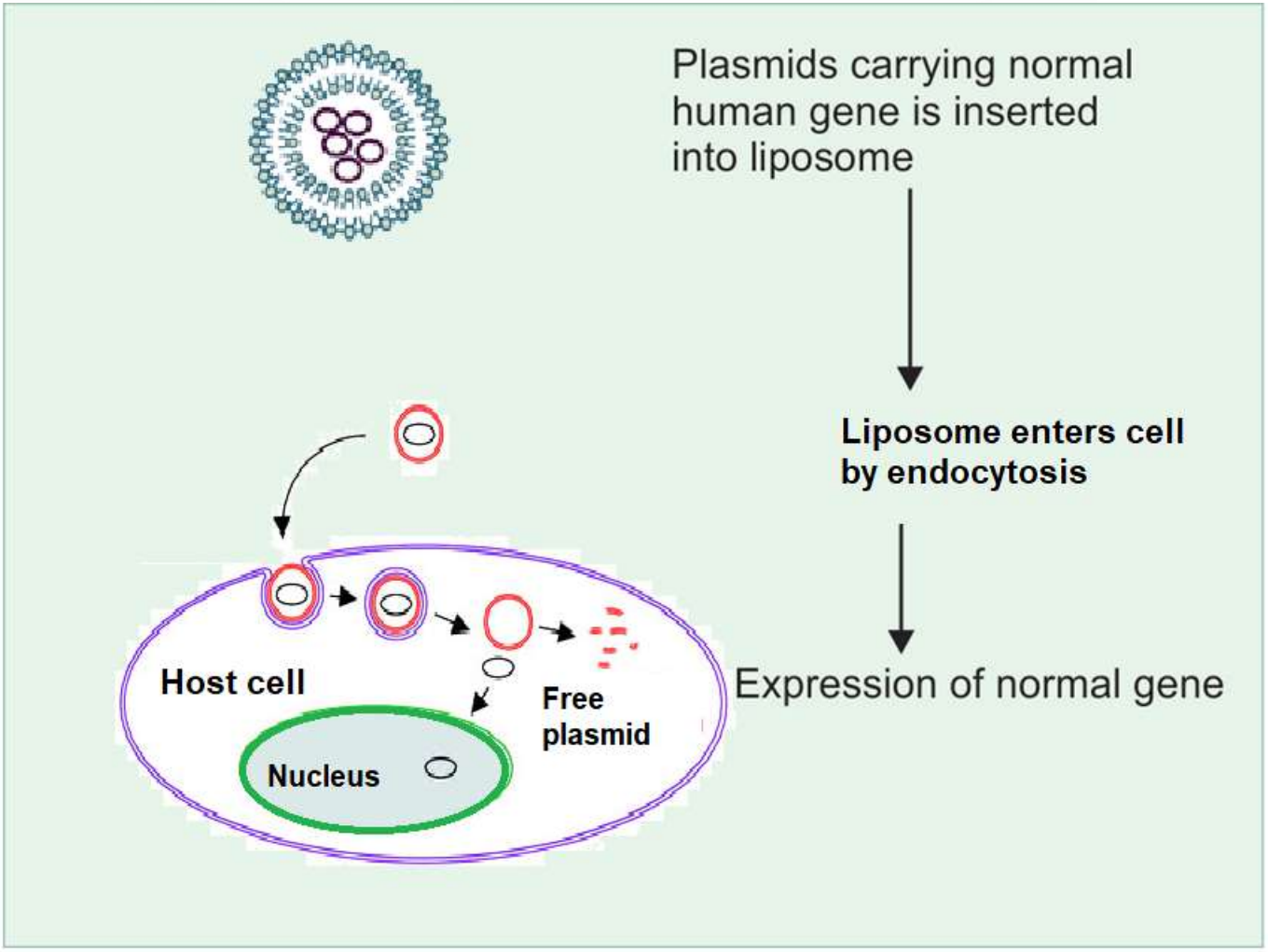
Naked viral RNA has to get protein coat to enable them to enter into human host cell. So, defective virus is put into packaging cells, which provide the viral coat

Virus particles with protein coat. These are infective (can enter into human cells); but cannot replicate or produce disease

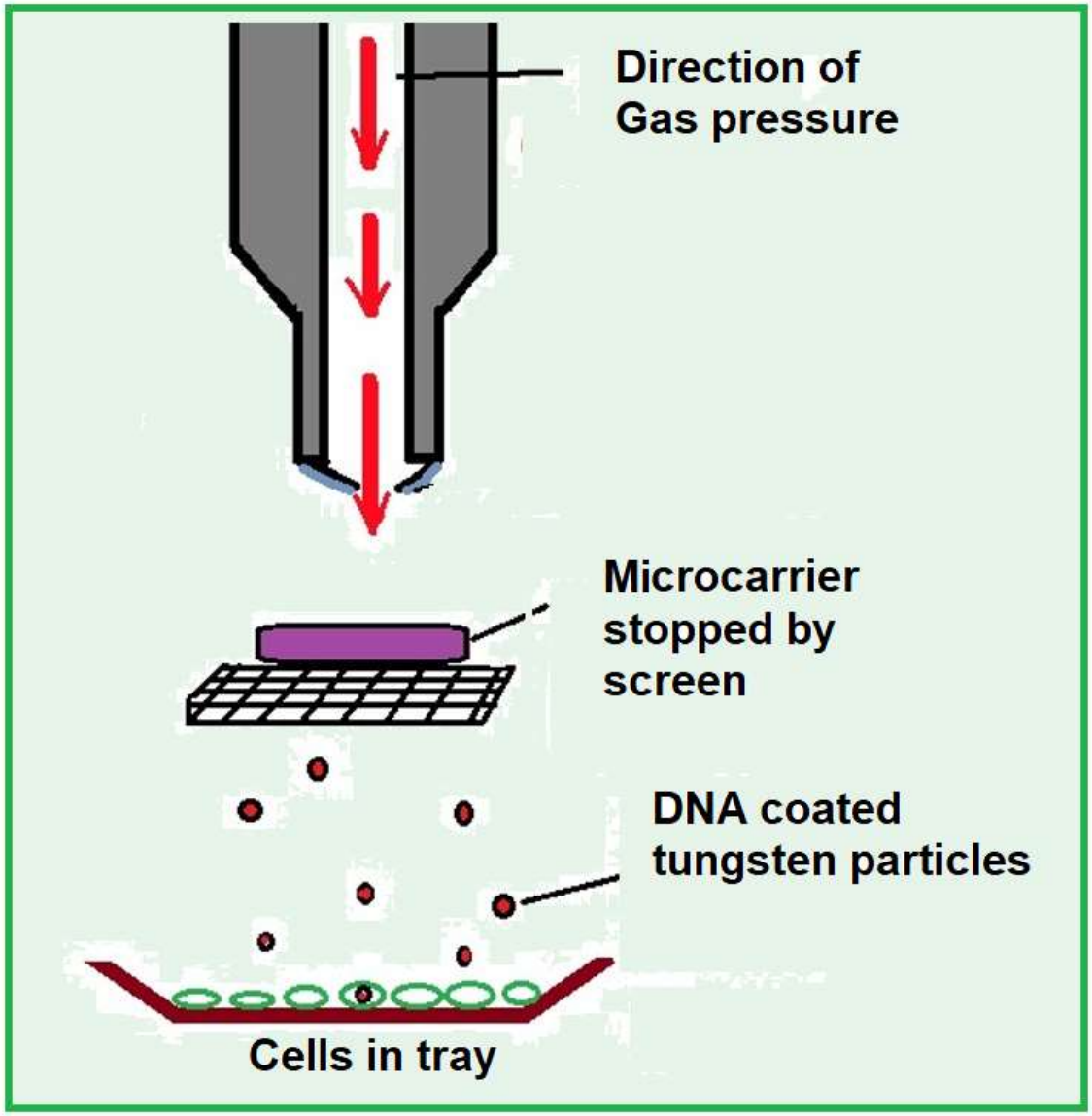
Gene Transfer by retroviral vector, continued



Gene Transfer by Plasmid liposome



Gene Transfer by Gene Gun Method



Tungsten particles are coated with plasmid DNA, and accelerated by helium pressure discharge. This enables particles to penetrate the target tissues. It is quick, and could be used in almost all tissues. The cellular damage and transient gene expression are the drawbacks.

Success Stories of Gene Therapy

Disease	Gene transferred by
1. Severe combined immunodeficiency (SCID)	Adenosine deaminase enzyme in chromosomes 13 and 20 into lymphocytes by retrovirus
2. Duchenne muscular dystrophy (DMD)	Dystrophin gene on the short arm of X chromosome; by retrovirus
3. Cystic fibrosis (CF)	CFTR gene on chromosome 7 to bronchial epithelium; adenovirus
4. Familial hyper-cholesterolemia	LDL receptor gene on chromosome 19 to hepatocytes; retrovirus
5. Hemophilia	A and B genes for factor VIII and IX into fibroblasts; retrovirus
6. Cancer	Activation of p53 (tumor suppressor gene) by liposome

Success Stories of Gene Therapy

Disease	Gene transferred by
7. Leukemia	The patient's own immune cells were removed and treated with a virus that altered them to recognize a protein present on the surface of the cancer cells.
8. Leber's Hereditary Optic Neuropathy	Introducing the gene for the enzyme (NADH dehydrogenase) using an adeno virus vector directly to the retina.
9. Beta thalassemia	Blood stem cells were taken from the patient's bone marrow and treated with a retrovirus to transfer a copy of the beta-globin gene. The modified stem cells were returned to his body.

Success Stories of Gene Therapy

Disease	Gene transferred by
10. Sickle cell disease	Blood stem cells were taken from the patient's bone marrow, treated with a retrovirus, and the modified stem cells were returned to his body.
11. Parkinson's disease	Retroviral vector to introduce three genes into cells in a small area of the brain. These genes gave cells that do not normally make dopamine the ability to do so

Obstacles to Success

The following limitations are encountered for gene therapy:

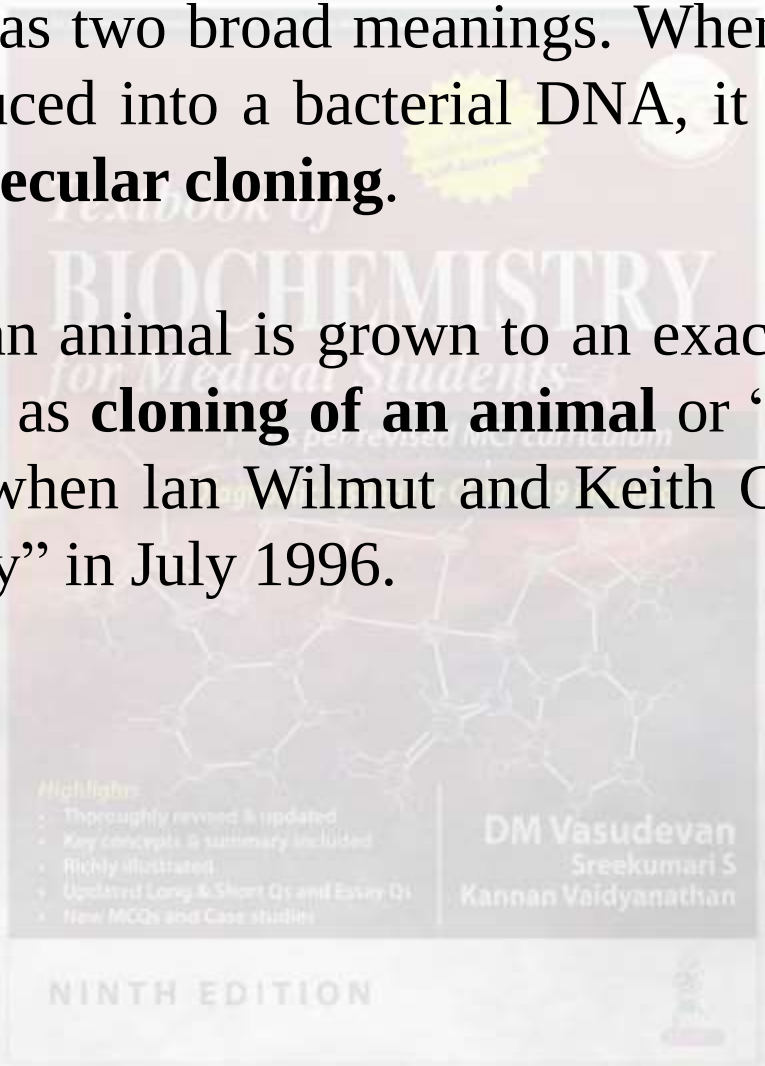
(a) Inconsistent results.
(b) Lack of ideal vector.
(c) Lack of targeting ability in the nonviral vectors.
(d) Death of the patient during the course of gene therapy for OTC (ornithine transcarbamoylase) deficiency was reported.
(e) Reactivation of retroviral vector due to illegitimate combination of the inserted gene leading to leukemia in the patient

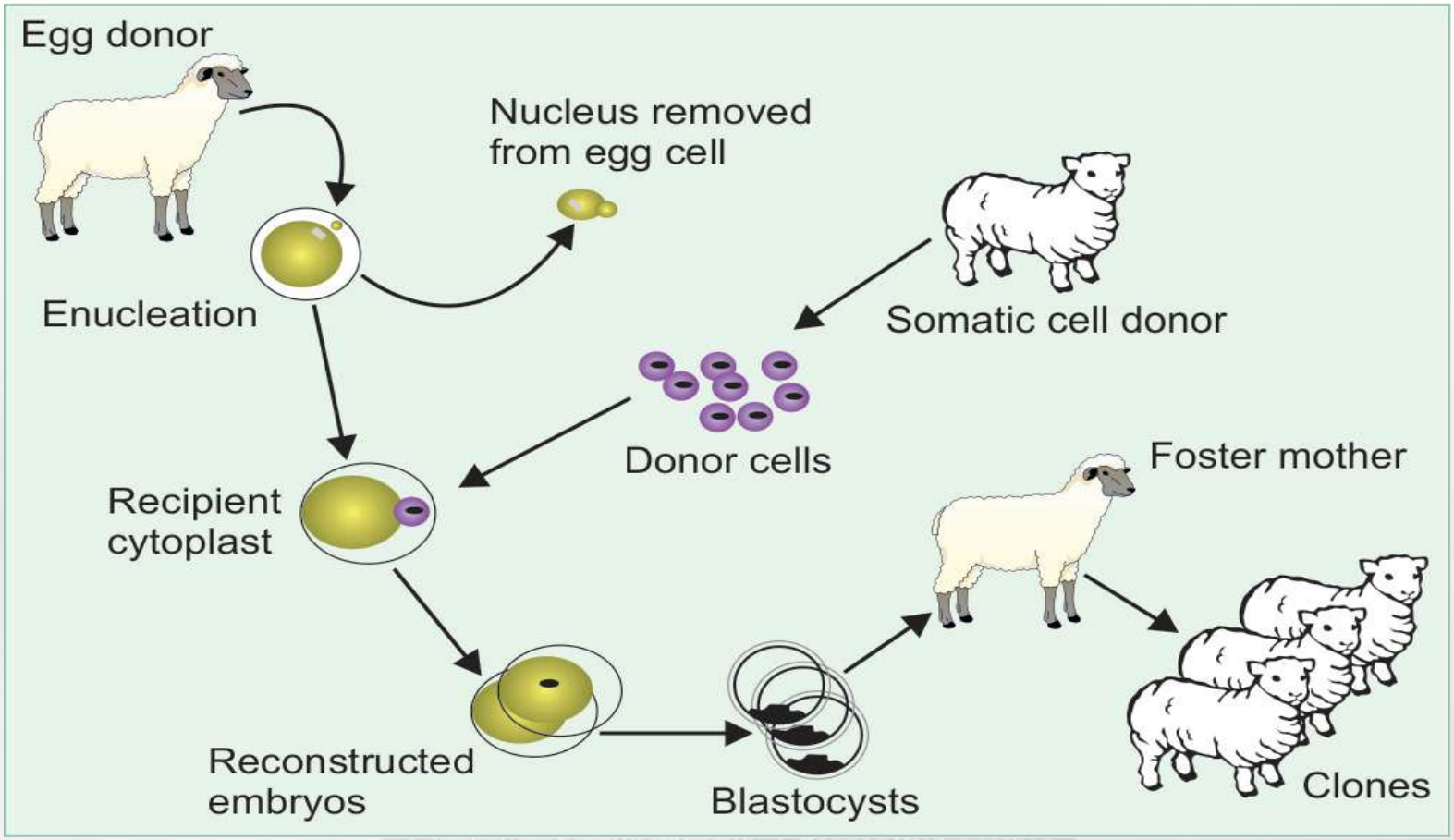
At present trials are on, but restricted with stringent protocols and follow up.

Animal Cloning

The term cloning has two broad meanings. When a gene of higher organism is introduced into a bacterial DNA, it is called "cloning of the gene" or **molecular cloning**.

When a cell from an animal is grown to an exact duplicate of that animal, it is known as **cloning of an animal** or "somatic cloning". It made big news when lan Wilmut and Keith Campbell cloned a sheep named "Dolly" in July 1996.





Cloning procedure adopted to make Dolly

Applications of Cloning of Animals and Plants

- Animals with genetically desirable traits could be bred more efficiently, e.g. cows yielding more milk.
- Biopharmaceuticals: By November 1998, the first goats were born, who were genetically engineered to produce milk containing antithrombin III. Any human protein could be introduced into the makeup of goat or cow
- and get the desired protein cheaply through the milk.
- Cloning is successfully employed in agriculture, to propagate plants such as rubber, banana, orchids, etc.
- Species threatening to become extinct could be reproduced easily.

Disadvantages of Cloning

Cloning will never replace selective breeding. Cloning halts any further progress. Cloning can produce the animals/plants with the same characteristics; new characteristics could not be developed.

The cloned animal and parent need not be exactly identical. First, mitochondrial DNA comes from the egg.

Second, DNA in an adult cell differs from the DNA in a fetal cell by the accumulated damages of a life time.

Thirdly, any animal is not just the product of its genes, but also of its environment, both in utero and after birth; this is especially so when higher organisms are concerned.

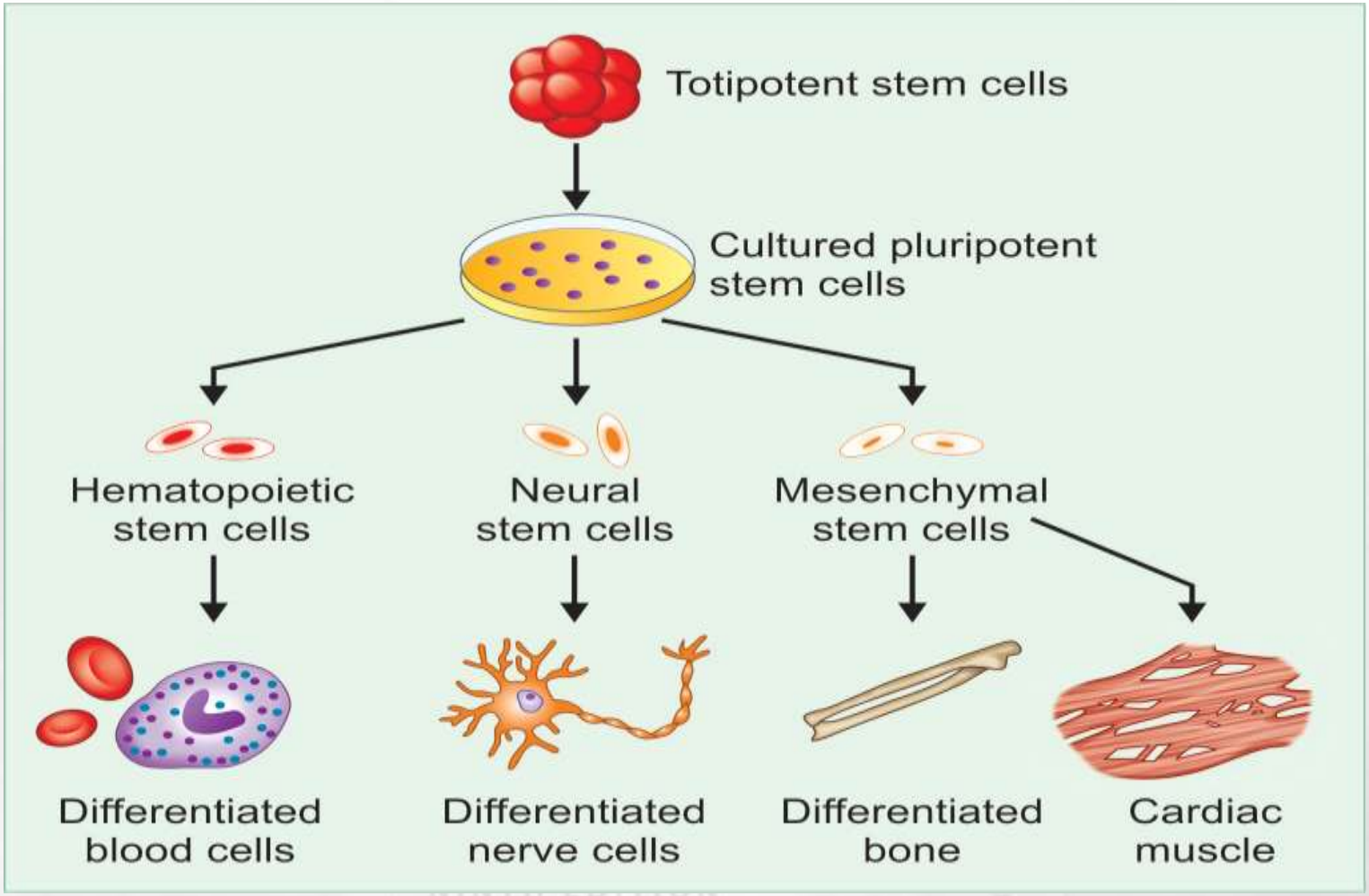
Stem Cells

Stem cells have the ability to divide for an indefinite period. They can give rise to a variety of specialized cell types. This phenomenon is known as **developmental plasticity**. Stem cells can be isolated from embryos, umbilical cord as well as from any other adult tissues. Plasticity is more for
embryonic stem cells.

Stem cells have the unique capacity to produce unaltered daughter cells (**renewal**) and also to generate specialized cells (**potency**). Stem cells may be capable of producing all types of cells of the organism (**totipotent**), or able to generate cells of the three germ layers (**pluripotent**), or able to produce only closely related cell types (**multipotent**), or may produce only one cell type (**unipotent**).

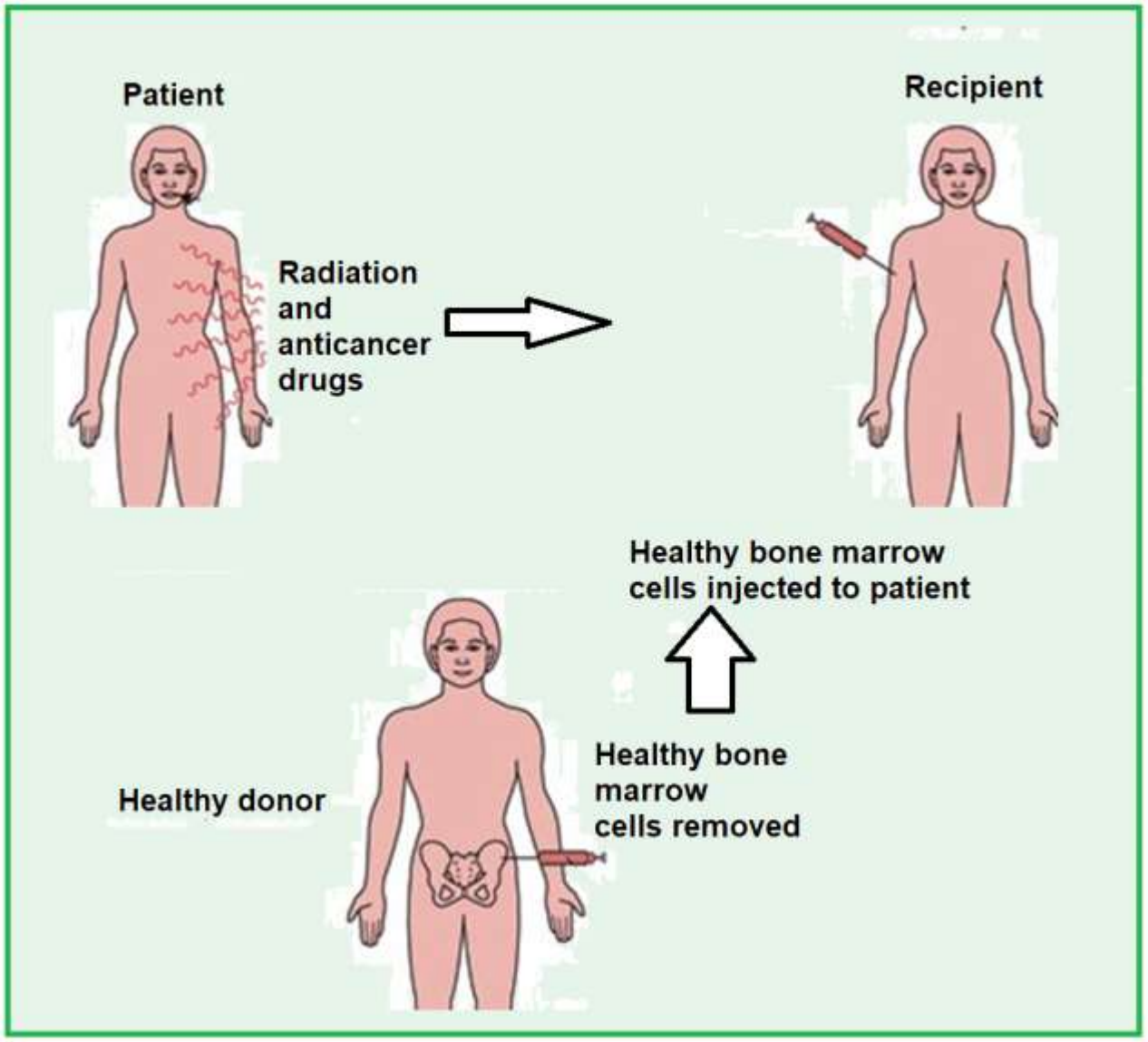
Active research is being done to utilize stem cells in the treatment of the diseases like stroke, brain injury, Alzheimer's disease, Parkinsonism, wound healing, myocardial infarction, muscular dystrophy, spinal cord injury, diabetes, and cancers.

Stem Cells



Differentiation of stem cells.

Allogenic bone marrow transplantation



Allogenic bone marrow transplantation

The patient is identified who has a potentially lethal disease and chemoradiotherapy should eradicate the disease. This will cause permanent destruction of hematopoietic stem cells. So, new normal hematopoietic stem cells can be infused into the patient's body. Such normal blood stem cells are harvested from a donor, who is matching with the patient. The sources of such stem cells are bone marrow, peripheral blood (usually after mobilization from the bone marrow) or umbilical cord blood. The patient is then nursed in a protective environment till the newly infused normal marrow cells start functioning. This usually takes about three to four weeks. Thus the patient is cured of his ailment. Indications for BMT are chronic myeloid leukemia (CML), thalassemia major, aplastic anemia, multiple myeloma and acute leukemias.