Recombinant DNA

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PART A

- Genetic engineering plays a very important role, not only in scientific research, but also in the diagnosis and treatment of disease.

rDNA is NOT Whole Animal Cloning

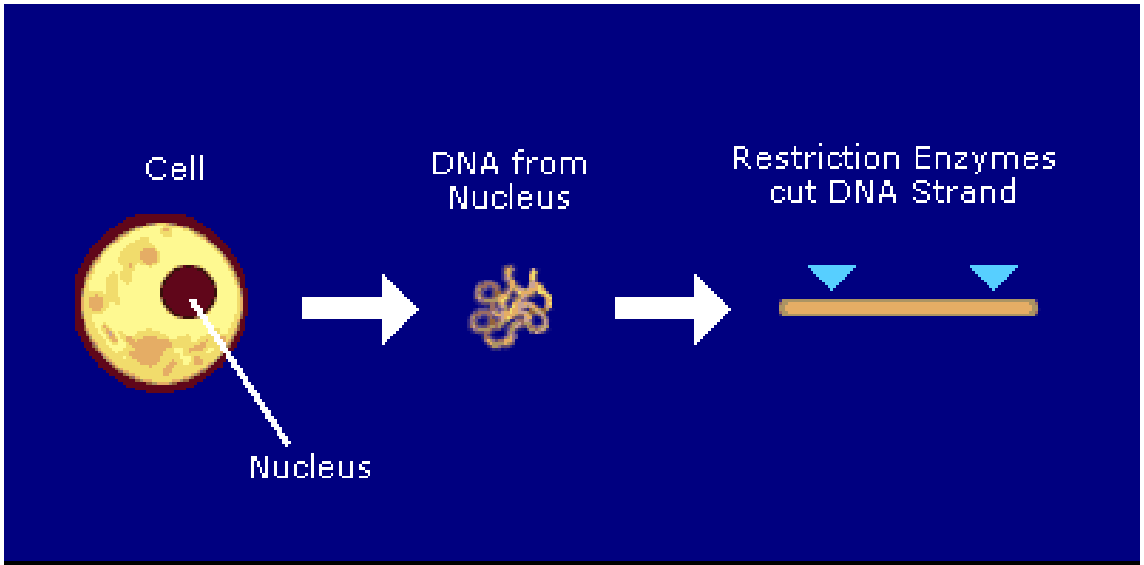
Recombinant DNA is a tool in understanding the structure, function, and regulation of genes and their products

- The objectives of Recombinant DNA technology include:
  - Identifying genes
  - Isolating genes
  - Modifying genes
  - Re-expressing genes in other hosts or organisms

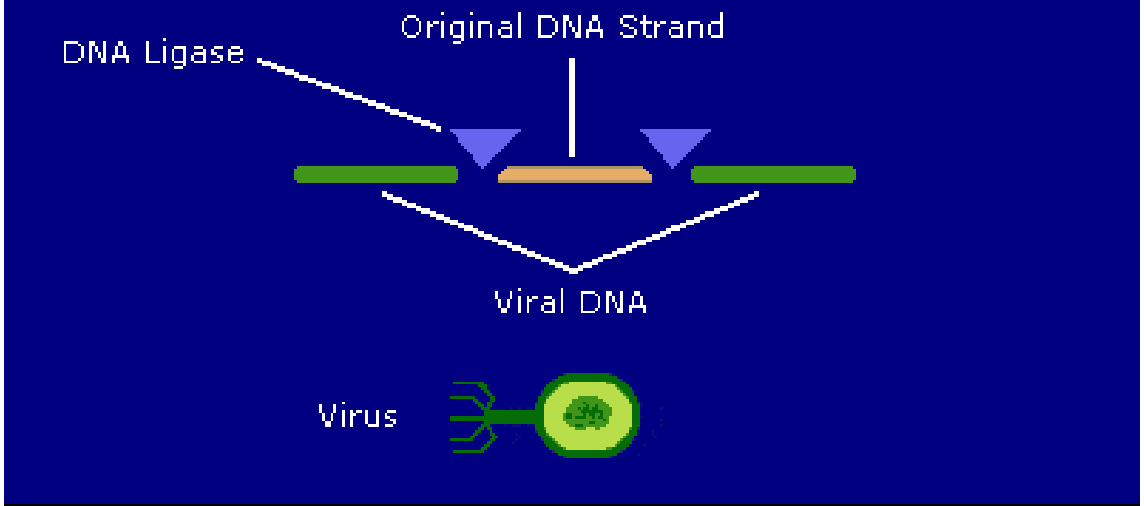
- These steps permit scientists and clinicians to:
  - Identify new genes and the proteins they encode
  - To correct endogenous genetic defects
  - To manufacture large quantities of specific gene products such as hormones, vaccines, and other biological agents of medical interest

Process Example

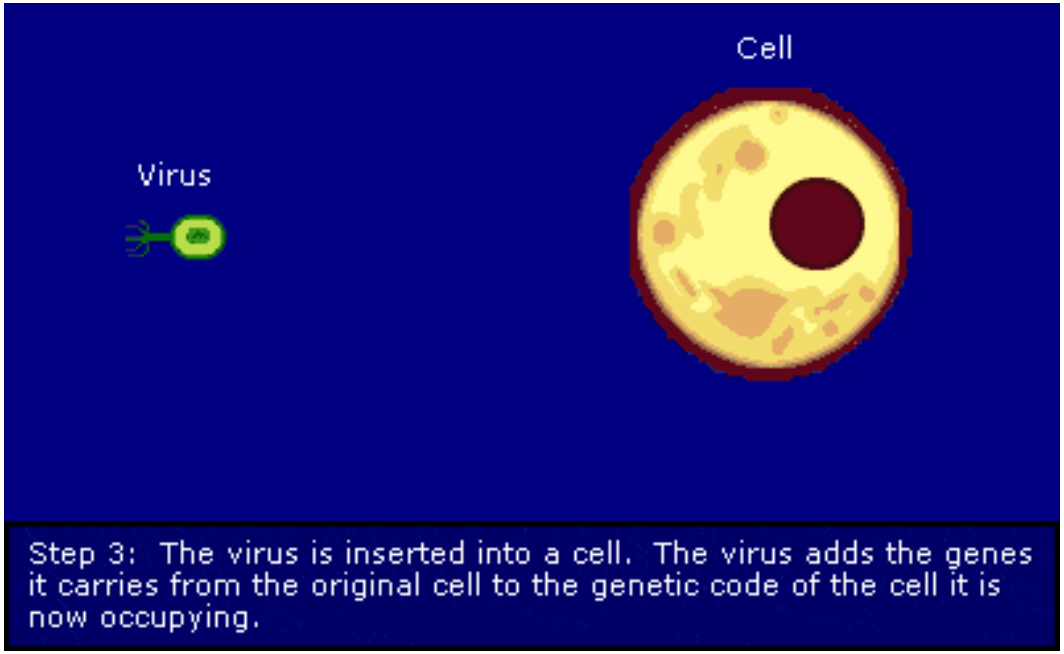
THINK ABOUT THIS ?
What is DNA Ligase Used for?
What is a Restriction Enzyme?
Why Are Virus' Used as Cloning Vectors?



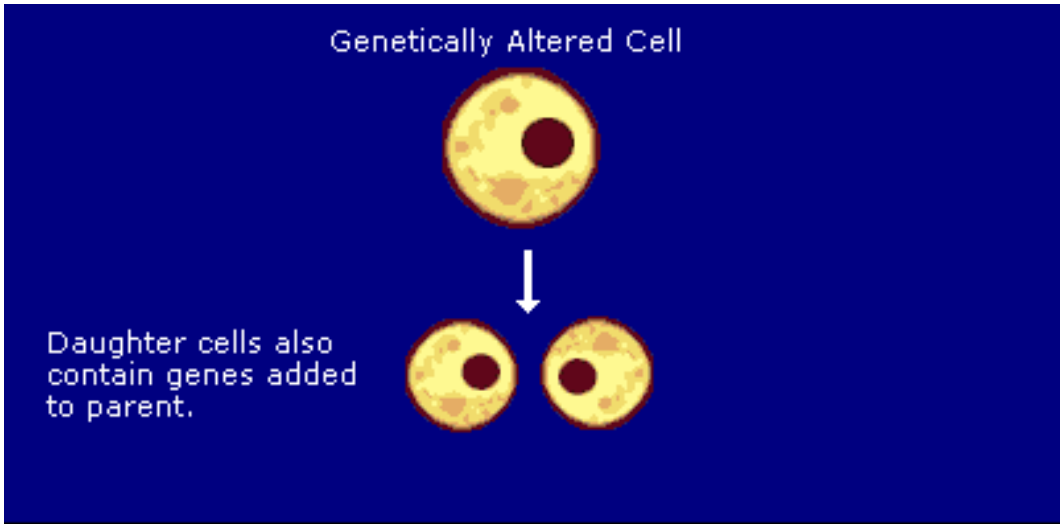
STEP 1: A restriction enzyme is used to cut a specific DNA strand from the DNA of a cell.



STEP 2: The cut DNA strand is inserted into a virus. DNA Ligase is used to bond the DNA fragment with the virus' DNA.



Step 3: The virus is inserted into a cell. The virus adds the genes it carries from the original cell to the genetic code of the cell it is now occupying.



Step 4: If the cell takes up the foreign gene, it passes the new genetic information to all daughter cells produced during division.

- Genetic engineering produces proteins that offer advantages over proteins isolated from other biological sources. These advantages include:
  - High purity
  - High specific activity
  - Steady supply
  - Batch-to-batch consistency

Steps in Synthesizing a Recombinant Protein:

- Recombinant technology begins with the isolation of a gene of interest. The gene is then inserted into a vector and cloned. A vector is a piece of DNA that is capable of independent growth; commonly used vectors are bacterial plasmids and viral phages. The gene of interest (foreign DNA) is integrated into the plasmid or phage, and this is referred to as recombinant DNA.

Steps in Synthesizing a Recombinant Protein:

- Before introducing the vector containing the foreign DNA into host cells to express the protein, it must be cloned. Cloning is necessary to produce numerous copies of the DNA since the initial supply is inadequate to insert into host cells.

Steps in Synthesizing a Recombinant Protein:

- Once the vector is isolated in large quantities, it can be introduced into the desired host cells such as mammalian, yeast, or special bacterial cells. The host cells will then synthesize the foreign protein from the recombinant DNA. When the cells are grown in vast quantities, the foreign or recombinant protein can be isolated and purified in large amounts.

- Recombinant DNA technology is not only an important tool in scientific research, but has also resulted in enormous progress in the diagnosis and treatment of certain diseases and genetic disorders in many areas of medicine.

Genetic engineering has permitted:

Identification of mutations:

People may be tested for the presence of mutated proteins that may be involved in the progression of breast cancer, retino-blastoma, and neurofibromatosis.

Genetic engineering has permitted:

Diagnosis of affected and carrier states for hereditary diseases:

Tests exist to determine if people are carriers of the cystic fibrosis gene, the Huntington's disease gene, the Tay-Sachs disease gene, or the Duchenne muscular dystrophy gene.

Genetic engineering has permitted:

Mapping of human genes on chromosomes:

Scientists are able to link mutations and disease states to specific sites on chromosomes.

Genetic engineering has permitted:

Transferring genes from one organism to another:

People suffering from cystic fibrosis, rheumatoid arthritis, vascular disease, and certain cancers may now benefit from the progress made in gene therapy.

Genetic engineering has permitted:

Isolation and alteration of genes:

Once gene modification becomes successful, alteration of genes to produce a more functional protein than the endogenous protein may become possible, opening up the route of gene therapy.

Genetic engineering has permitted:

Performing structure and function analyses on proteins:

Researchers may now employ rational drug design to synthesize drug compounds that will be efficacious and selective in treating disease.

Genetic engineering has permitted:

Isolation of large quantities of pure protein:

Insulin, growth hormone, follicle-stimulating hormone, as well as other proteins, are now available as recombinant products. Physicians will no longer have to rely on biological products of low purity and specific activity from inconsistent batch preparations to treat their patients.

PART B
Case Study: The Use of Recombinant DNA to Produce Human Insulin

Why synthesize human insulin?

- Patients' immune systems do not produce antibodies against human insulin as they do with bovine or porcine insulin
- Projected decline in the production of animal-derived insulin
- Need for a more reliable and sustainable method of obtaining the product

Why is insulin needed?

- Protein hormone produced by beta cells of islets of Langerhans in the pancreas
- Regulates blood sugar by allowing uptake of glucose from bloodstream into body cells
- Patients with diabetes have insufficient or impaired production of insulin

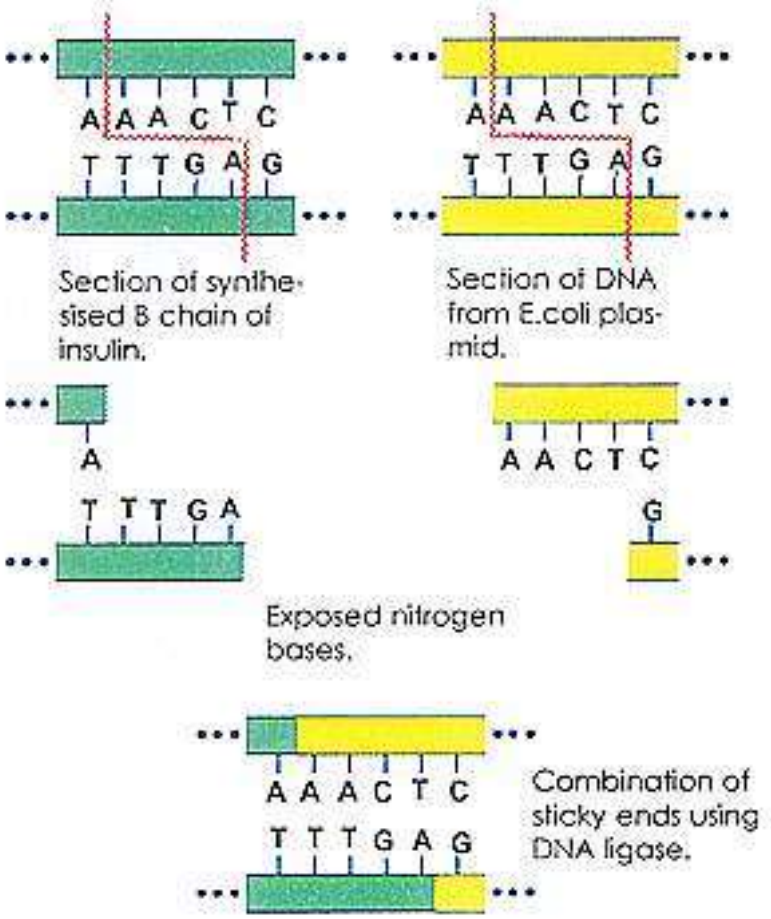
Structure of Insulin

- Two polypeptide chains; one with 21 amino acids and the second with 30 amino acids
- Chains are linked via a disulfide bond
- Gene encoding the insulin protein is found on chromosome 11

Recombinant DNA Technique

- Restriction enzymes used to cut out insulin gene and to cut a bacterial

  (*E. coli*) plasmid at the same "sticky ends"



Recombinant DNA Technique

- Mutant strains of *E. coli* used to avoid bacteria attacking "foreign" genes
- Insert insulin gene next to *E. coli*

  B-galactosidase gene which controls transcription
- Bacterial cells replicate and make copies of insulin gene

Recombinant DNA Technique

- Insulin protein is purified (B-galactosidase removed)
- Chains are mixed and disulfide bridges form
- Yeast cells provide a sterile growth medium
- Final product is Humulin - chemically identical to human insulin

Possible Complications of Using Human Insulin

- hypoglycemia (low blood sugar) tends to be more common than with animal insulin

Part 6

Cloning Vectors

Cloning Vectors

- A vector is used to amplify a single molecule of DNA into many copes. A DNA fragment must be inserted into a cloning vector. A cloning vector is a DNA molecule that has an origin of replication and is capable of replicating in a bacterial cell.

- Most vectors are genetically engineered plasmids or phages. There are also cosmid vectors, bacterial artificial chromosomes, and yeast artificial chromosomes.

Plasmid Cloning Vectors

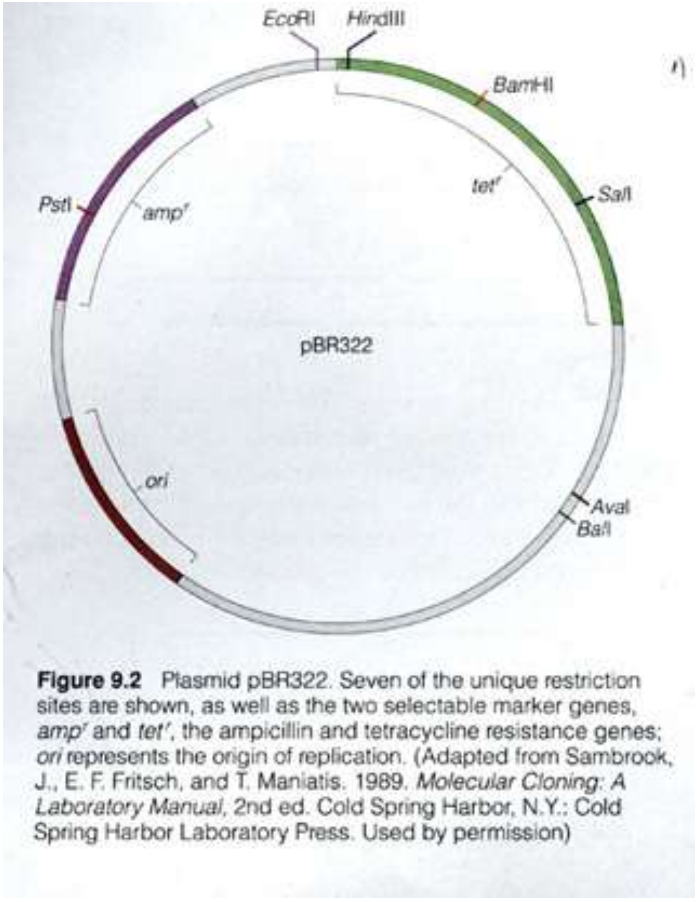


Figure 9.2 Plasmid pBR322. Seven of the unique restriction sites are shown, as well as the two selectable marker genes, *amp^r* and *tet^r*, the ampicillin and tetracycline resistance genes; *ori* represents the origin of replication. (Adapted from Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. Used by permission)

- Plasmids are circular, double-stranded DNA molecules that exist in bacteria and in the nuclei of some eukaryotic cells.
- They can replicate independently of the host cell. The size of plasmids ranges from a few kb to near 100 kb
- Can hold up to 10 kb fragments
- Plasmids have an origin of replication, antibiotic resistance genes as markers, and several unique restriction sites.
- After culture growth, the clone fragment can be recovered easily. The cells are lysed and the DNA is isolated and purified.
- A DNA fragment can be kept indefinitely if mixed with glycerol in a –70 degrees C freezer.

Plasmid Polylinkers and Marker Genes for Blue-White screening

Figure 9.8 Blue-white screening on medium with ampicillin, X-gal, and IPTG. Blue colonies contain nonrecombinant plasmids. White colonies contain recombinant plasmids and can be isolated directly from this plate.

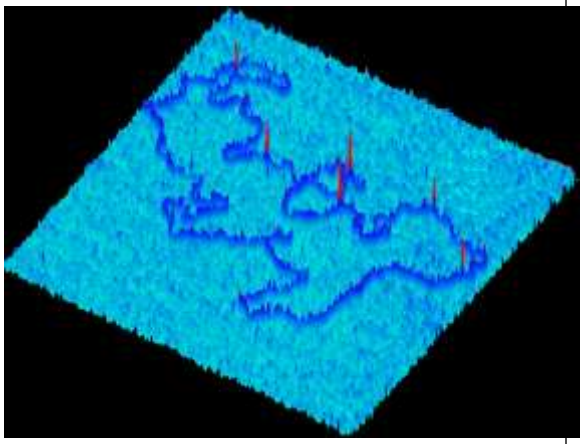
- A vector usually contains a sequence (polylinker) which can recognize several restriction enzymes so that the vector can be used for cloning a variety of DNA samples.
- Colonies with recombinant plasmids are white, and colonies with nonrecombinant plasmids are blue.
- Example: pUC19
- Resistant to ampicillin, has (amp^r gene)
- Contains portion of the lac operon which codes for beta-galactosidase.
- X-gal is a substrate of beta-galactosidase and turns blue in the presence of functional beta-galactosidase is added to the medium.
- Insertion of foreign DNA into the polylinker disrupts the lac operon, beta-galactosidase becomes non-functional and the colonies fail to turn blue, but appear white.

Phage Cloning Vectors

- Fragments up to 23 kb can be may be accommodated by a phage vector
- Lambda is most common phage
- 60% of the genome is needed for lytic pathway.
- Segments of the Lambda DNA is removed and a stuffer fragment is put in.
- The stuffer fragment keeps the vector at a correct size and carries marker genes that are removed when foreign DNA is inserted into the vector.
- Example: Charon 4A Lambda
- When Charon 4A Lambda is intact, beta-galactosidase reacts with X-gal and the colonies turn blue.
- When the DNA segment replaces the stuffer region, the lac5 gene is missing, which codes for beta-galactosidase, no beta-galactosidase is formed, and the colonies are white.

Cosmid Cloning Vectors

- Fragments from 30 to 46 kb can be accommodated by a cosmid vector.
- Cosmids combine essential elements of a plasmid and Lambda systems.
- Cosmids are extracted from bacteria and mixed with restriction endonucleases.
- Cleaved cosmids are mixed with foreign DNA that has been cleaved with the same endonuclease.
- Recombinant cosmids are packaged into lambda caspids
- Recombinant cosmid is injected into the bacterial cell where the rcosmid arranges into a circle and replicates as a plasmid. It can be maintained and recovered just as plasmids.



Shown above is a 50,000 base-pair long DNA molecule bound with six EcoRI molecules, and imaged using the atomic force microscope. This image clearly indicates the six EcoRI "sites" and allows an accurate restriction enzyme map of the cosmid to be generated.

http://homer.ornl.gov/cbps/afmimaging.htm

Bacterial Artificial Chromosomes(BACs) and Yeast Artificial Chromosomes(YACs)

- BACs can hold up to 300 kbs.
- The F factor of E.coli is capable of handling large segments of DNA.
- Recombinant BACs are introduced into E.coli by electroportation (a brief high-voltage current). Once in the cell, the rBAC replicates like an F factor.
- Example: pBAC108L
- Has a set of regulatory genes, OriS, and repE which control F-factor replication, and parA and parB which limit the number of copies to one or two.
- A chloramphenicol resistance gene, and a cloning segment.

- YACs can hold up to 500 kbs.
- YACs are designed to replicate as plasmids in bacteria when no foreign DNA is present. Once a fragment is inserted, YACs are transferred to cells, they then replicate as eukaryotic chromosomes.
- YACs contain: a yeast centromere, two yeast telomeres, a bacterial origin of replication, and bacterial selectable markers.
- YAC plasmid→Yeast chromosome
- DNA is inserted to a unique restriction site, and cleaves the plasmid with another restriction endonuclease that removes a fragment of DNA and causes the YAC to become linear. Once in the cell, the rYAC replicates as a chromosome, also replicating the foreign DNA.

Part D
HIV Gene Delivery System?

The characteristics that make some retroviruses dangerous pathogens, are the very characteristics that make them an excellent gene transfer system

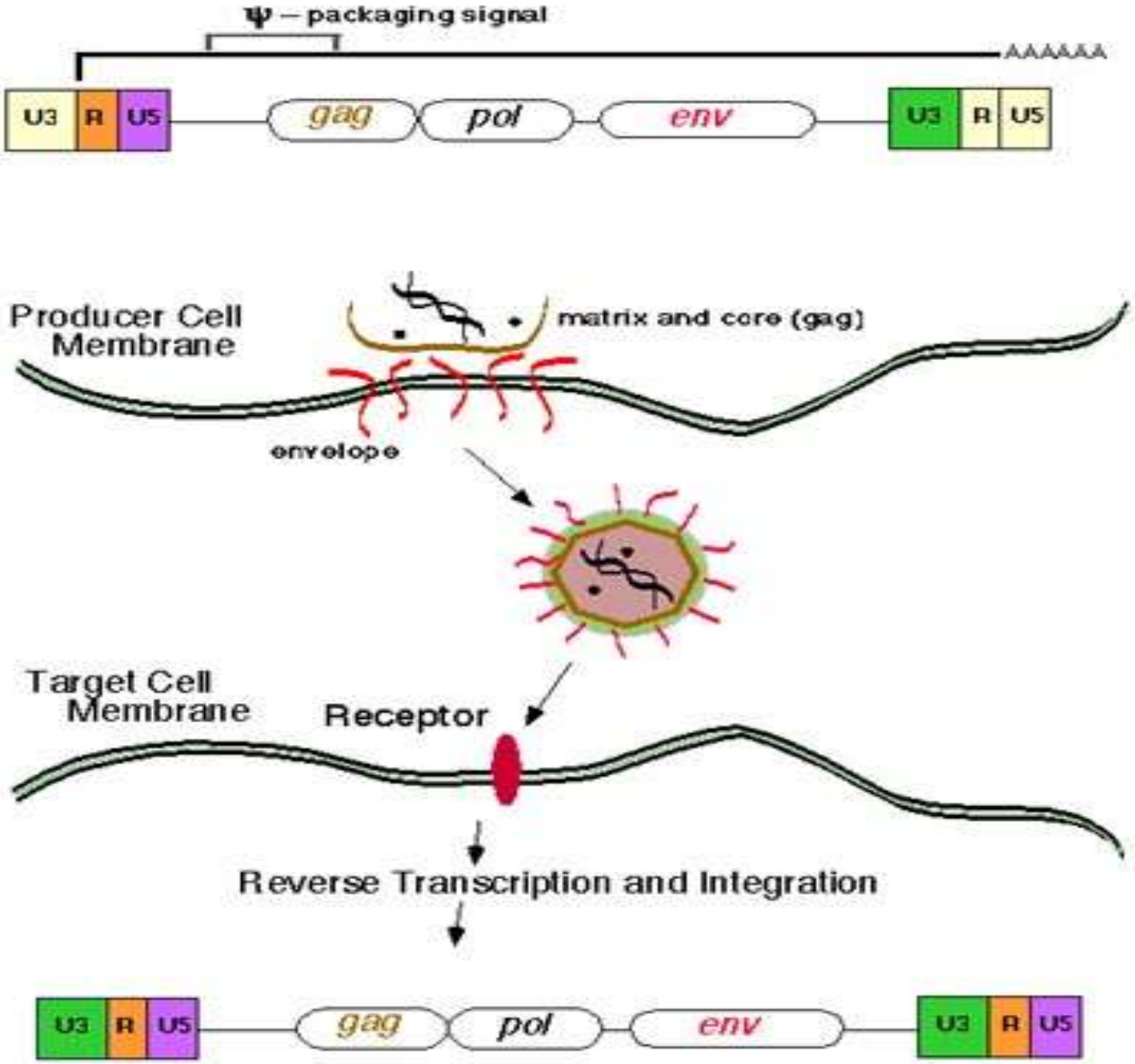
Retrovirus Characteristics

Retroviruses exist as proviruses in the hosts genome

Retroviruses have powerful promoters

Retroviral genomes can accommodate changes to its configuration

Retroviruses are the only animal viruses that integrate into the hosts genome



The virus that causes aids may one day be used in gene therapy

WHY?

Because it is an lentivirus

What is a Lentivirus?

Lentiviruses are a subfamily of retroviruses – HIV is a lenitivirus

Why is a lentivirus necessary?

Lentiviruses can introduce a gene of interest into cells that do not divide – simple retroviruses cannot

This ability makes them ideal for a delivery system because most of our cells, like hemopoietic stem cells, do not divide

Why use HIV?

A genetically stripped down amalgam of HIV components can be fashioned with a molecular switch system that turns them off in response to a common antibiotic

This type of control allows doctors to control gene expression in people who are treated with gene therapy - If something goes wrong, the expression can be turned off

Adenoviruses are often used as a vector in gene therapy research but they do not have the capacity to integrate their genome into the hosts genome

The advantage to using a retrovirus is that you don’t lose the genomic sequence that is incorporated into the host DNA following cell division

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