What is recombinant DNA technology?

Molecular biology technique borrows many of its principles based on observation of the microbial world. In the early 1960s, before the advent of gene cloning, studies of genes often relied on indirect or fortuitous discoveries, such as the ability of bacteriophages to incorporate bacterial genes into their genomes. A huge contribution to recombinant DNA technology is based on the products and processes followed by the microbes.

The cornerstone of most molecular biology technologies is the gene. To facilitate the study of genes, they can be isolated and amplified. One method of isolation and amplification of a gene of interest is to clone the gene by inserting it into another DNA molecule that serves as a vehicle or vector that can be replicated in living cells. When these two DNAs of different origin are combined, the result is a recombinant DNA molecule. Although genetic processes such as crossing-over technically produce recombinant DNA, the term is generally reserved for DNA molecules produced by joining segments derived from different biological sources. The recombinant DNA molecule is placed in a host cell, either prokaryotic or eukaryotic. The host cell then replicates (producing a clone), and the vector with its foreign piece of DNA also replicates. The foreign DNA thus becomes amplified in number, and following its amplification can be purified for further analysis.

Insights from bacteriophage lambda (l) cohesive sites

In 1962, Allan Campbell noted that the linear genome of bacteriophage λ forms a circle upon entering the host bacterial cell, and a recombination (breaking and rejoining) event inserts the phage DNA into the host chromosome. Reversal of the recombination event leads to normal excision of the phage DNA. Further analysis revealed that phage λ had short regions of single-stranded DNA whose base sequences were complementary to each other at each end of its linear genome. These single-stranded regions were called “cohesive” (cos) sites.

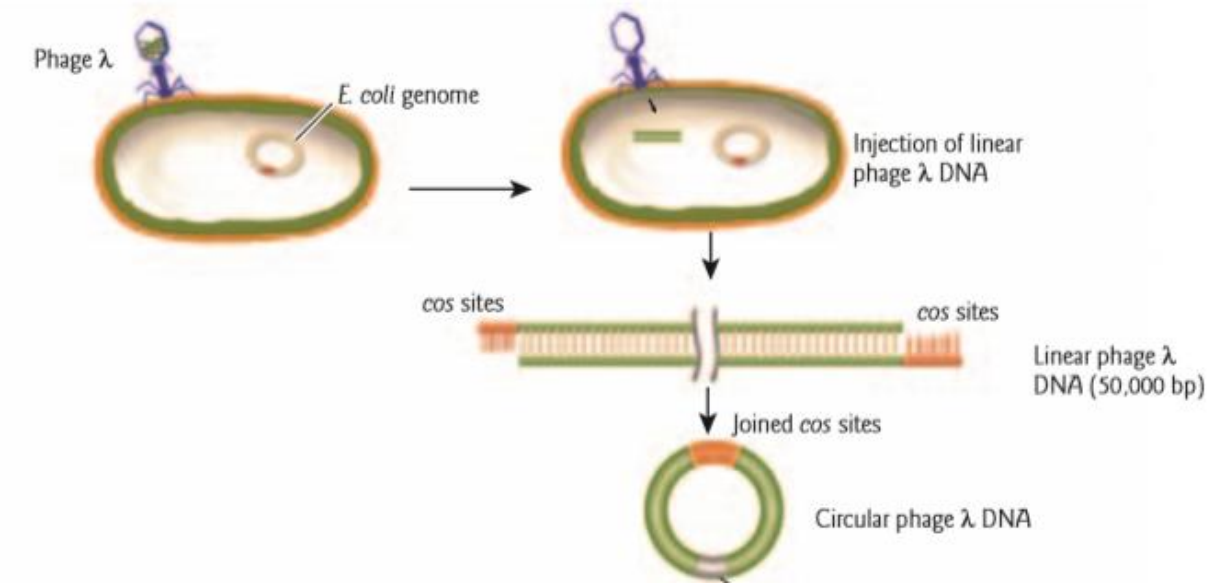

Figure 1- Cloning (insertion) of viral DNA into bacterial (host) cell. The cohesive ends are complementary to each other and thus gain a circular form in the host after insertion.

Complementary base pairing of the cos sites allowed the linear genome to become a circle within the host bacterium. The idea of joining DNA segments by "cohesive sites" became the guiding principle for the development of genetic engineering. With the molecular characterization of restriction and modification systems in bacteria, it soon became apparent that the ideal engineering tools for making cohesive sites on specific DNA pieces were already available in the form of restriction endonucleases. Hamilton Smith and co-workers demonstrated unequivocally that restriction endonucleases cleave a specific DNA sequence. The activity of cutting by restriction endonucleases, mostly, produced cohesive ends. Restriction endonucleases (also referred to simply as restriction enzymes) received their name because they restrict or prevent viral infection by degrading the invading nucleic acid.

Modification system.

At the time, it was known that methyl groups were added to bacterial DNA at a limited number of sites. Most importantly, the location of methyl groups varied among bacterial species. Arber and colleagues were able to demonstrate that modification consisted of the addition of methyl groups to protect those sites in DNA sensitive to attack by a restriction endonuclease. Methyl-modified target sites are no longer recognized by restriction endonucleases and the DNA is no longer degraded. Once established, methylation patterns are maintained during replication. In contrast, foreign DNA that is unmethylated or has a different pattern of methylation than the host cell DNA is degraded by restriction endonucleases.

Cutting and joining DNA

Two major categories of enzymes are important tools in the isolation of DNA and the preparation of recombinant DNA: restriction endonucleases and DNA ligases. Restriction endonucleases recognize a specific, rather short, nucleotide sequence on a double-stranded DNA molecule, called a restriction site, and cleave the DNA at this recognition site or elsewhere, depending on the type of enzyme. DNA ligase joins two pieces of DNA by forming phosphodiester bonds. To increase the efficiency of the reaction, researchers often use the enzyme terminal deoxynucleotidyl transferase to modify the blunt ends (no cohesive ends). For example, if a single-stranded poly(dA) tail is added to DNA fragments from one source, and a single stranded poly(dT) tail is added to DNA from another source, the complementary tails can hydrogen bond. Recombinant DNA molecules can then be created by ligation.

It should be appreciated that the entire framework of molecular cloning rests heavily on the contributions made by the microbial world.

Molecular cloning

The basic procedure of molecular cloning involves a series of steps:

First, the DNA fragments to be cloned are generated by using restriction endonucleases.

Second, the fragments produced by digestion with restriction enzymes are ligated to other DNA molecules that serve as vectors. Vectors can replicate autonomously (independent of host genome replication) in host cells and facilitate the manipulation of the newly created recombinant DNA molecule.

Third, the recombinant DNA molecule is transferred to a host cell. Within this cell, the recombinant DNA molecule replicates, producing dozens of identical copies known as clones. As the host cells replicate, the recombinant DNA is passed on to all progeny cells, creating a population of identical cells, all carrying the cloned sequence.

Finally, the cloned DNA segments can be recovered from the host cell, purified, and analyzed in various ways.

Vector DNA

Cloning vectors are carrier DNA molecules. Four important features of all cloning vectors are that they: (i) can independently replicate themselves and the foreign DNA segments they carry; (ii) contain a number of unique restriction endonuclease cleavage sites that are present only once in the vector; (iii) carry a selectable marker (usually in the form of antibiotic resistance genes or genes for enzymes missing in the host cell) to distinguish host cells that carry vectors from host

cells that do not contain a vector; and (iv) are relatively easy to recover from the host cell. There are many possible choices of vector depending on the purpose of cloning.

Plasmid DNA as a vector

Plasmids are naturally occurring extrachromosomal, double-stranded circular DNA molecules that carry an origin of replication and replicate autonomously within bacterial cells. The plasmid vector pBR322, constructed in 1974, was one of the first genetically engineered plasmids to be used in recombinant DNA. Plasmids are named with a system of uppercase letters and numbers, where the lowercase "p" stands for "plasmid." In the case of pBR322, the BR identifies the original constructors of the vector (Bolivar and Rodriquez), and 322 is the identification number of the specific plasmid. pUC18 is a derivative of pBR322.

Cloning

Plasmid vectors are modified to contain a specific antibiotic resistance gene and a multiple cloning site (also called the polylinker region) which has a number of unique target sites for restriction endonucleases. Cutting the circular plasmid vector with one of these enzymes results in a single cut, creating a linear plasmid. A foreign DNA molecule, referred to as the "insert," cut with the same enzyme, can then be joined to the vector in a ligation reaction. Ligations of the insert to vector are not 100% productive, because the two ends of a plasmid vector can be readily ligated together, which is called self-ligation. But, if the vector is joined with a foreign insert, the 5'-phosphate is provided by the foreign DNA. Another strategy involves using two different restriction endonuclease cutting sites with noncomplementary sticky ends. This inhibits self-ligation and promotes annealing of the foreign DNA in the desired orientation within the vector.

Transformation: transfer of recombinant plasmid DNA to a bacterial host

The ligation reaction mixture of recombinant and nonrecombinant DNA described in the preceding section is introduced into bacterial cells in a process called transformation. The permeable "competent" cells are then mixed with DNA to allow entry of the DNA into the bacterial cell. Successfully transformed bacteria will carry either recombinant or nonrecombinant plasmid DNA. Multiplication of the plasmid DNA occurs within each transformed bacterium. A single bacterial cell placed on a solid surface (agar plate) containing nutrients can multiply to form a visible colony made of millions of identical cells. As the host cell divides, the plasmid vectors are passed on to progeny, where they continue to replicate. Numerous cell divisions of a single transfomed bacteria result in a clone of cells (visible as a bacterial colony) from a single parental cell. This step is where "cloning" got its name. The cloned DNA can then be isolated from the clone of bacterial cells.

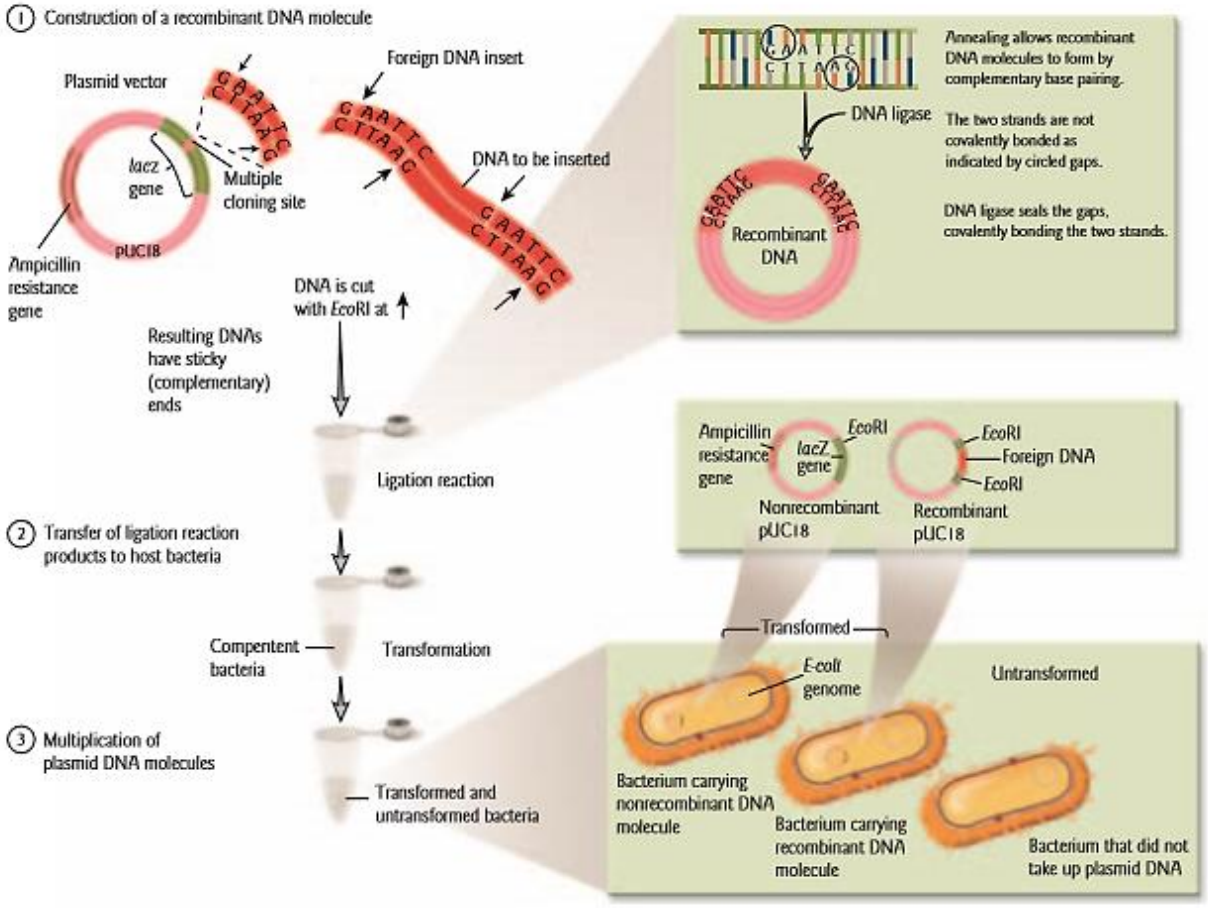

Figure 2- Production of recombinants and transformation

Recombinant selection

What needs to be included in the medium for plating cells so that nontransformed bacterial cells are not able to grow at all? The answer depends on the particular vector, but in the case of pUC18, the vector carries a selectable marker gene for resistance to the antibiotic ampicillin. Ampicillin, a derivative of penicillin, blocks synthesis of the peptidoglycan layer that lies between the inner and outer cell membranes of E. coli. Ampicillin does not affect existing cells with intact cell envelopes but kills dividing cells as they synthesize new peptidoglycan. The ampicillin resistance genes carried by the recombinant plasmids protects against ampicillin. Nontransformed cells contain no pUC18 DNA, therefore they will not be antibiotic-resistant, and their growth will be inhibited on agar containing ampicillin. Transformed bacterial cells may contain either nonrecombinant pUC18 DNA (selfligated vector only) or recombinant pUC18 DNA (vector containing foreign DNA insert). Both types of transformed bacterial cells will be ampicillin-

resistant. Further screening may be done to separate out the recombinant from non-recombinant types.

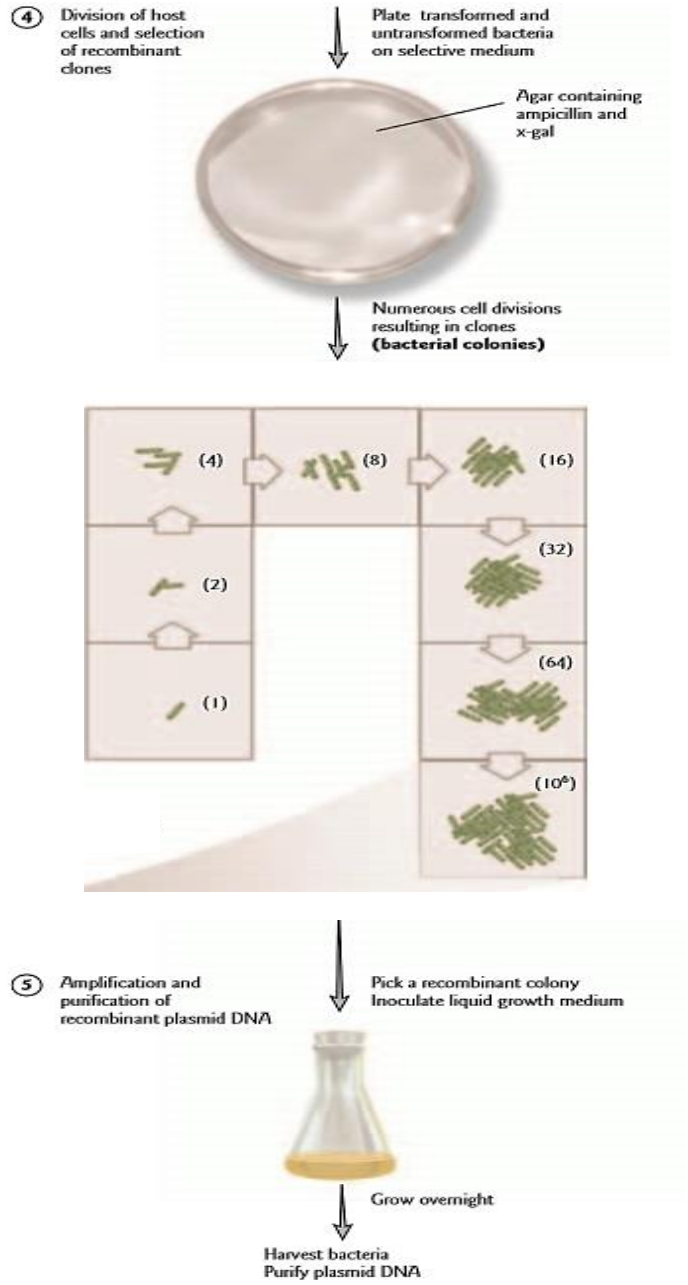

Figure 3- Recombinant selection on a selective media

The final step is purification of the plasmid DNA and can be further used for various purposes.

Application of molecular cloning

Molecular cloning provides scientists with an essentially unlimited quantity of any individual DNA segments derived from any genome. This material can be used for a wide range of purposes,

including those in both basic and applied biological science. A few of the more important applications are i) To study the genome organization and gene expression; ii) Production of recombinant proteins; iii) Transgenic organisms/genetically modified organisms (GMOs); Although most GMOs are generated for purposes of basic biological research (transgenic mouse), a number of GMOs have been developed for commercial use, ranging from animals and plants that produce pharmaceuticals or other compounds (pharming), herbicide-resistant crop plants, and fluorescent tropical fish (GloFish) for home entertainment. iv) Gene therapy; Gene therapy involves supplying a functional gene to cells lacking that function, with the aim of correcting a genetic disorder or acquired disease. Gene therapy can be broadly divided into two categories. The first is alteration of germ cells, that is, sperm or eggs, which results in a permanent genetic change for the whole organism and subsequent generations. This "germ line gene therapy" is considered by many to be unethical in human beings. The second type of gene therapy, "somatic cell gene therapy", is analogous to an organ transplant. In this case, one or more specific tissues are targeted by direct treatment or by removal of the tissue, addition of the therapeutic gene or genes in the laboratory, and return of the treated cells to the patient. Clinical trials of somatic cell gene therapy began in the late 1990s, mostly for the treatment of cancers and blood, liver, and lung disorders.

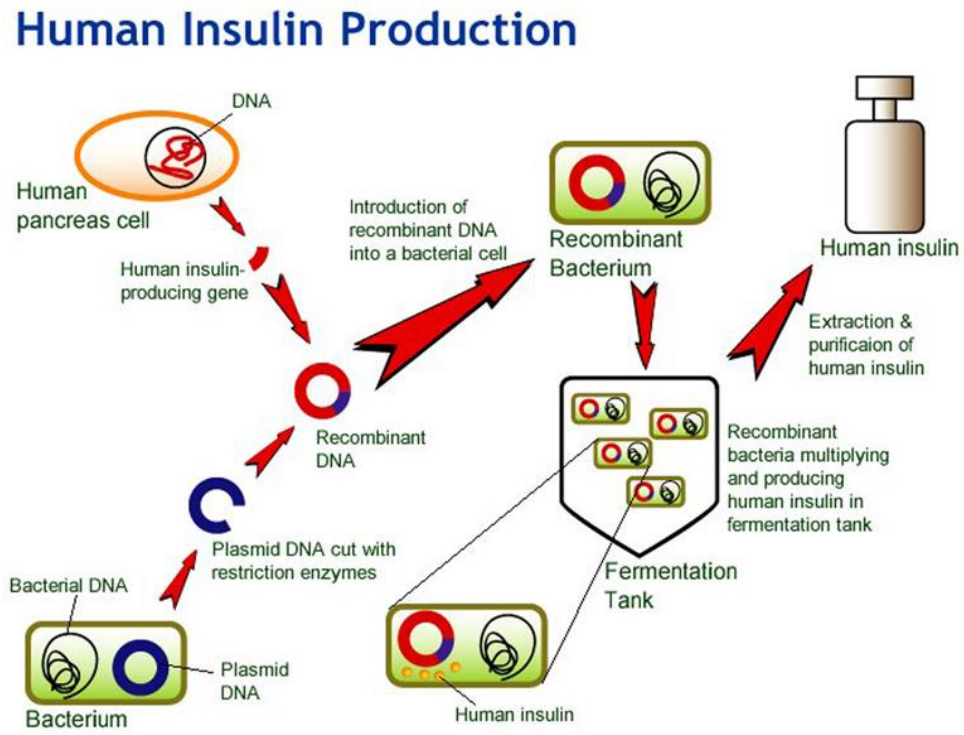

Figure 4- Human insulin production using molecular cloning. The production of human insulin chains in bacterium is a classic example of the application of molecular cloning for improving human life. Initially, bovine and porcine sources were used for the purpose of human insulin production. However, this gave rise to various ethical and medical problems. These problems are successfully overcome by recombinant DNA technology to produce insulin in bacteria.