

## CHAPTER SIX

# Gene Cloning

### Introduction

Genetic engineering or the genetic manipulation is defined as the *in vitro* synthesis for new forms or new arrangements of DNA in such way as to allow the incorporation or propagation of altered genetic condition to continue in nature. The *in vitro* manipulation of DNA is called recombinant DNA technology. Broadly, genetic engineering means the manipulation of genes under controlled laboratory conditions. The importance of genetic engineering as a modern technique stems from many reasons, such as it allows the isolation and studying of individual genes in large quantities and in complete purity. These genes are possible to be expressed on and it's possible to be reused in the cells whether they are of one type or different types.

The experiments of genetic engineering achieved many goals that a lot of fields of biology were unable to achieve, such as the induction of bacteria to synthesize hormones and the cost effective and rare biological products. The best known example for such precious compounds is insulin, human growth hormone, and rennin. All of these three compounds were extracted before the discovery of genetic engineering from animals, such as cows, pigs and calves, or from human bodies. The hope of the workers in genetic engineering is to use these experiments in the future to correct some disorders that originated from mutations such as sickle cell and thalassemia (gene therapy). These manipulations also aim to provide new features such as in herbicides, the frost resistance for plants, or to produce bigger and best farm animals. Most of these huge achievements for this science are originated from the enormous development in gene cloning successfully in the recipient cells. Therefore, in this chapter, we will review the fundamental tools to show us how to clone certain gene step by step until we reach to the successful expression of this gene into the host cells.

### The Successive Tools for Gene Cloning

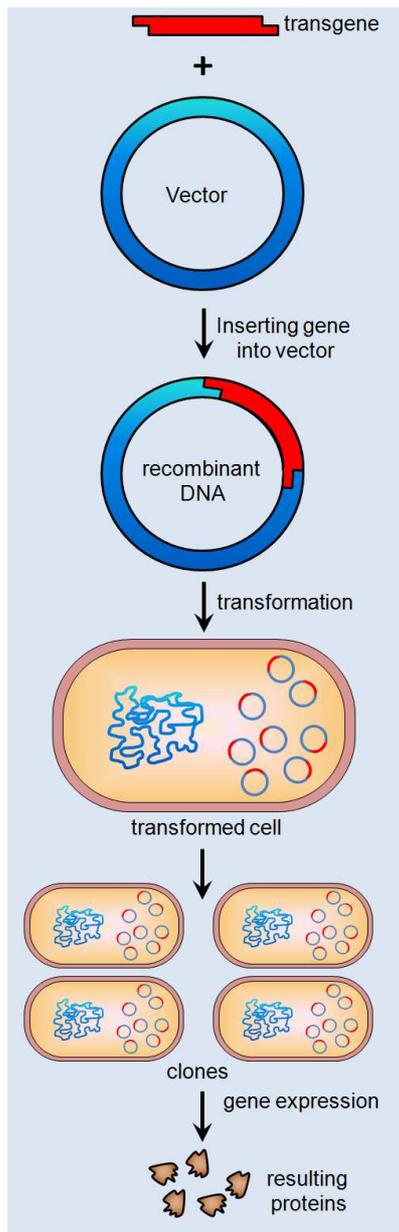
Many biological steps are used to achieve the manipulation of the genetic material to perform to clone certain gene. But before getting to them we have to know that many DNA manipulation experiments require enormous quantities (copies) of a particular DNA piece. These steps are called "gene cloning", because of the production of homologous copies of the original DNA fragment. To perform this practically, it's usually to insert the gene into a certain vector to form the recombinant DNA molecule. The vector acts as a vehicle to present the gene into the host cell, to direct its

replication in its required form, and to enable it to express about itself in some cases to produce the hybrid protein (figure 6.1). The host cell carrying the gene-containing vector produces progeny all of which contain the inserted gene. These identical cells are called "clones". In the transformed host cell and its clones, the inserted gene is expressed about itself in some cases in such a way it's transcribed and translated into the recombinant proteins.

Classically, to clone certain gene, certain tools should be provided through which the genetic material that have this gene is isolated. Now, several tools are provided by which the researcher can easily isolate the genetic material. Then, this gene must be separated from the rest of the genes in the isolated genetic material. To do so, many restriction endonucleases are available which cut the gene of interest of its either sides. Then, the necessary tools that connect this gene with the suitable gene vector must be provided (such as ligases – see below). This is done after cutting the vector with the same restriction enzyme that has been usually used in the separation of the gene of interest. After formation of the recombinant molecule, other tools must be provided that introduce this recombinant molecule into the host cells. Finally, the tools that enable the researcher to know or select the clones that have these recombinant molecules must be provided. These tools constitute the essential basics in gene cloning that are not difficult to be understood by the inexperienced reader. Therefore, we will review these tools sequentially to complete the picture to us about the cloning of the gene.

### **First; Tools of Separation of DNA containing the Desired Gene**

Before we clone any gene in the experiments of genetic engineering we have to obtain it in satisfied quantities. This is done by three methods differ with the difference of characteristics of the desired piece of DNA and the purpose of its cloning. In the first method, it's possible to isolate the organism's entire DNA, then, its fragments are digested into several pieces, the desired fragment of interest isolated, and eventually cloned. Or, it's possible to clone all the fragments by a suitable vector, than each clone is tested for the presence of the desired gene. Or the desired DNA fragment can be directly synthesized in order to be cloned afterward.



**Figure (6.1);** general scheme for gene cloning. After isolating the gene of the DNA fragment to be cloned, this fragment is ligated with the suitable gene vehicle to form the recombinant DNA molecule. This molecule is introduced into the recipient cells by transformation. The recipient cells express about the products of the cloned gene.

## Second; Tools of cutting and Sealing DNA Fragments

After obtaining the genetic material that has the desired gene with high purity, we need now to isolate this gene from the rest of the genetic material of the donor cell. To do so, we have to provide a tool that "scissor" this gene from its edges. These endonuclease enzymes that occur naturally and function in the restriction and modification system in bacteria, are quickly became known as restriction enzymes.

With accumulating years, the utilization of restriction enzymes in the experiments of genetic engineering becomes popular, in such away the researcher that is working in this field in the late 1990s could choose from thousands of commercially available highly purified restriction enzymes for cut it molecule at any desired site. But, before the number get so high, researcher found a successful way to avoid confusion about restriction enzymes nomination.

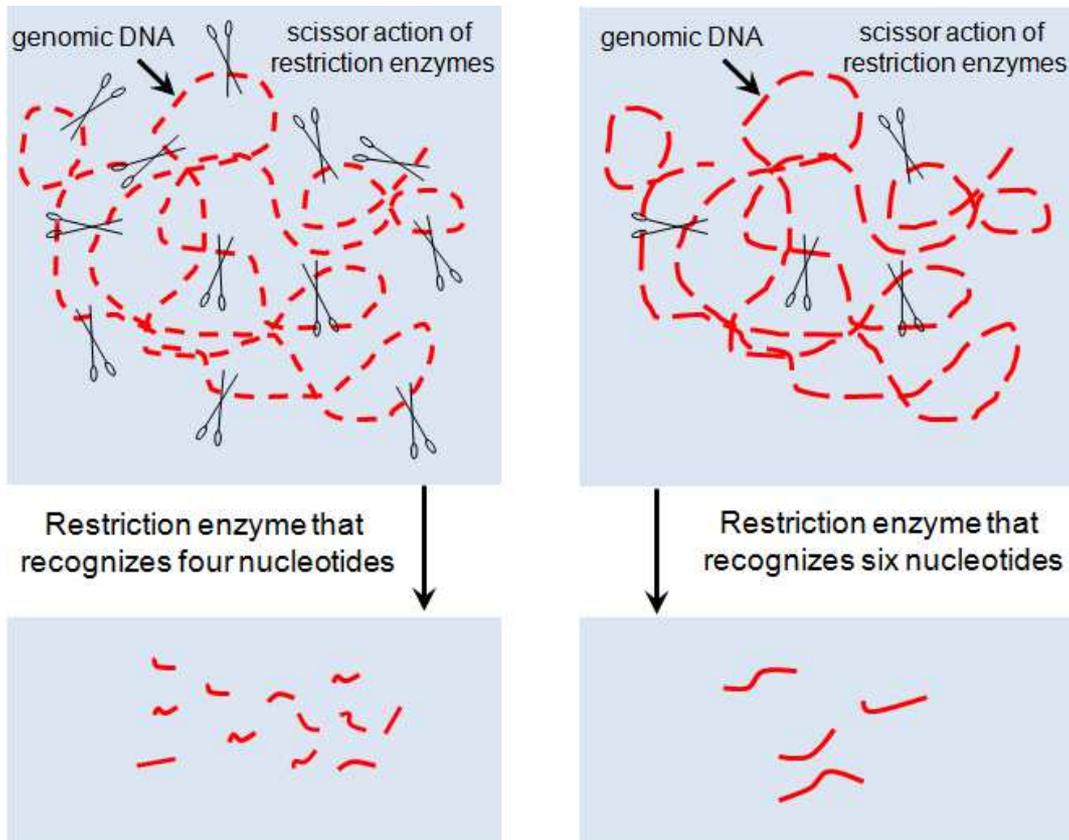
These nominations were derived from the name of the bacterium from which they were isolated. For example, the *Eco* RI restriction enzyme was named from *Escherichia coli*, *Bam*HI was named from *Bacillus amyloliquefaciens*. The three letters of the name of the restriction enzyme consists of; The first letter of the enzyme name is the first letter of the bacterium's genus name (such as *E*), followed by the first two letters of its species (such as *co*), then a letter representing the bacterial strain, and finally a Roman numeral that signifies the order in which this enzyme was discovered. This is applied in organisms that have more than one restriction enzyme (such as *Eco*RI or *Eco*RII). Not that the first three letter of the name of enzyme are written in italic style.

Each restriction enzyme cuts (or nicks) DNA at a specific base sequence, called the recognition sequence, which is can be varied from 4 into 16 or 20 base pairs. As the number of base pairs of the recognition sequence

increased, as the number of cuts made in DNA usually decreased. If we postulate that the DNA is distributed randomly in the DNA molecule, hence, we can calculate how much the enzyme cut the DNA molecule.

A significant notion must be known, which is that for each position of DNA molecule, there are four possibilities, (A, C, G, or T). Therefore, the restriction enzymes that recognize four nucleotides sequence, they will cut once per each 256 bps (or  $4^4$ ). Whereas, other enzymes recognize six nucleotide sequences, so, they cut once per each 4096 bps (or  $4^6$ ) (figure 6.2).

After the identity of restriction enzymes has been known, it must be know how restriction enzymes identify the recognition sequences that available at DNA in order to cut them. So how do enzymes find their recognition sequences? Scientist have found that restriction enzymes adopt specific three dimensional shapes that progress along nonspecific sequences in a DNA helix, “scanning” until reaching their specific recognition sequence. Most restriction enzymes recognize and cut at a palindrome DNA sequences. These sequences represent the substrates exposed to attack by restriction endonucleases.



**Figure (6.2).** The reverse relationship between the frequency of cleavage of DNA strands by restriction enzymes with the number of recognized nucleotides at the site of cleavage. The restriction enzyme that recognize four nucleotides sequences cuts the DNA with more frequency and produces smaller fragments compared with the restriction enzyme that recognizes six nucleotides sequences.

In the English language, a palindrome is when the letters of a sentence spell the same words whether they are read forward or backward; for example, “Madam, I’m Adam” or “Ma is a nun as I am”. As for the DNA, the

palindromic site is a sequence of bases in the DNA duplex which has the same reading either from forward or from reverse direction (from 5' to 3' or the opposite). For example, the sequence GAATTC is "palindromic" since both sequences of the either strands have the same reading whether from G end or from C end (figure 6.3).

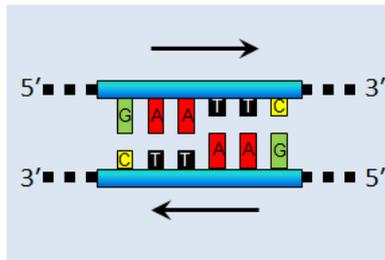


Figure (6.3); an example about the palindromic sequence (the sequence that is cut by *EcoRI*).

The most known example for restriction enzymes is *EcoRI*, which is synthesized by the bacterium *E. coli* from the strain RY13. This enzyme attacks the nucleotide sequence GAATTCCTTAAG. It can be noticed that this palindrome is symmetrical about its center. The restriction enzyme *EcoRI* makes single stranded cuts called "nicks", between A and G of both strands and this opens the circular DNA molecule.

There are different known types of restriction endonucleases, which nicks different regions of the DNA molecule. And on the basis of mode of nicking of the specialized DNA sequence, there are two types of restriction enzymes; the first group nicks straightly across the DNA duplex, to produce blunt end DNA. The other type of restriction enzymes nicks the DNA from the center of the palindromic sequence but between the same nitrogen bases on the opposite strands. This leaves one or more overhang base from each strand. These ends called sticky ends (figure 6.4). They are called in this name because their formation for hydrogen bonds with their complementary bases that are nicked by the same enzyme. The sticky ends are especially important in the construction of the recombinant DNA molecules.

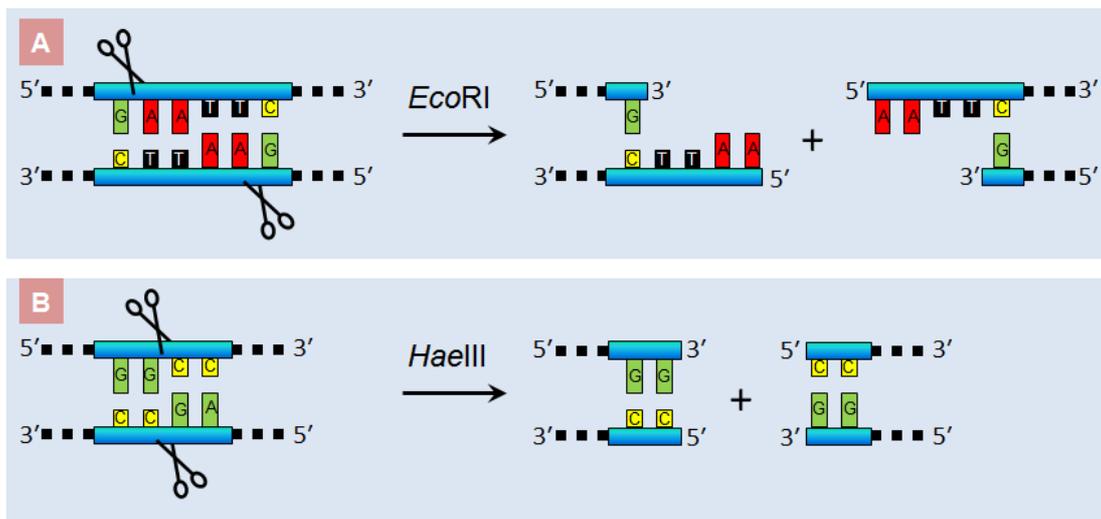
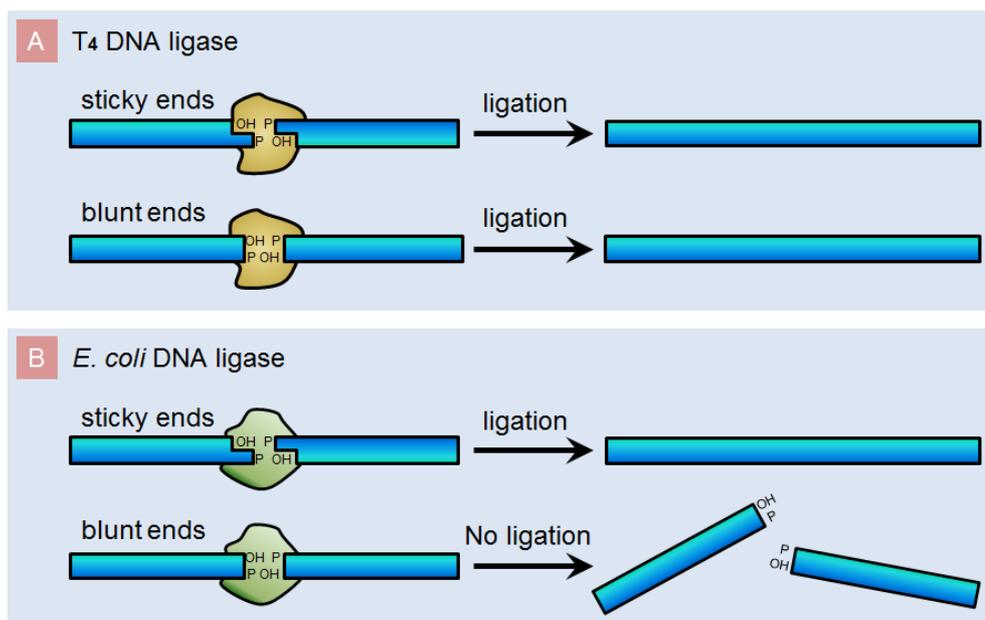


Figure (6.4); types of restriction enzymes according to the mode of nicking. (A) An example about sticky ends restriction enzymes. (B) An example about blunt ends restriction enzymes.

Since the single stranded ends that generated in the nicking site are complementary, they can be corrected with each other. Thus, the generated DNA fragments by the same endonuclease can be joined back easily together by forming base pairs (figure 6.5).

After nicking the foreign DNA molecules by suitable restriction enzymes, they usually joined with the suitable gene vehicles. This is done by ligation enzymes or DNA ligase. The joining of the two strands is done a sealing enzyme that is also called "gluing agent" to join the separated nucleic acids together. This ligase joins the DNA fragments covalently. In intracellular level, the DNA ligase acts during DNA replication, which joins Okazaki fragments together to construct the lagging strands (see chapter three). Two requirements must be met for DNA ligase to do its task. First, the molecules must be the correct substrates, that is, they must possess 3'-hydroxyl and 5'-phosphate groups. Second, the groups on the molecules to be joined must be properly positioned with respect to one another. Simply, if the DNA ligase found two DNA fragments annealed together from their termini, it seals them together. In the experiments of genetic engineering, the sticky ended DNA fragments tend to be aligned together for long period of time. Consequently, DNA ligase joins them efficiently. Since the blunt ended DNA fragments don't have any way to be joined together, they are far away from each other for most of time. Consequently, the sealing of blunt ends is very slow and requires a high concentration of DNA ligase, in addition to the high concentration of DNA fragments to be sealed. It's noteworthy that the bacterial DNA ligases are unable to join the blunt ends. Therefore, the T<sub>4</sub> DNA ligase that is originally derived from the bacteriophage T<sub>4</sub> is used in the ligation of the blunt ended DNA fragments (figure 6.5).



**Figure (6.5);** ligation of DNA fragments by DNA ligase in an energy required reaction. (A) the success of the sealing of blunt or sticky ended DNA termini by T<sub>4</sub> DNA ligase, (B) the success of the sealing of DNA termini by bacterial DNA ligase and the failure of sealing of blunt ended DNA termini by the same enzyme.

**Restriction Enzyme Digestion Protocol**

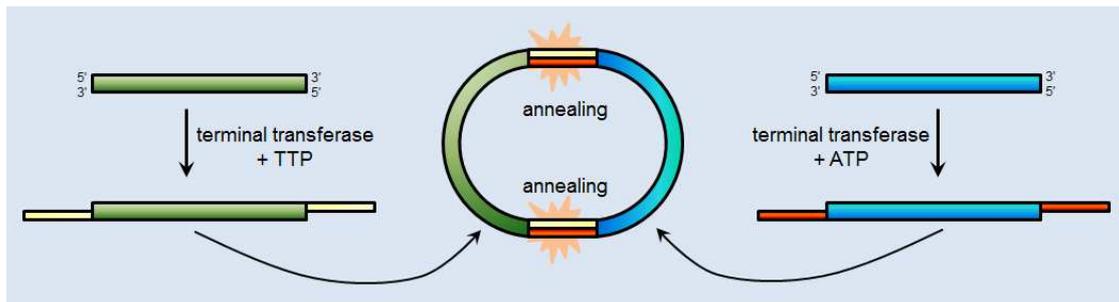
Before assembling the restriction digest, thoroughly mix each component to be added to the reaction and then centrifuge the tubes of reagents briefly to collect the contents in the bottom of the tube. The reaction components should also be mixed after addition of the enzyme to the digest. While high salt buffers and glycerol-containing reagents are difficult to mix, all solutions containing restriction enzymes must be mixed gently to avoid inactivating the enzyme.

An analytical scale restriction enzyme digest is usually performed in a volume of 20 $\mu$ l on 0.2–1.5 $\mu$ g of substrate DNA, using a two- to tenfold excess of enzyme over DNA. If an unusually large volume of DNA or enzyme is used, aberrant results may occur and may or may not be readily recognized. The following is an example of a typical RE digest. In a sterile tube, assemble in order:

sterile, deionized water	16.3 $\mu$ l
RE 10X Buffer	2 $\mu$ l
Acetylated BSA,	10 $\mu$ g/ $\mu$ l 0.2 $\mu$ l
DNA, 1 $\mu$ g/ $\mu$ l	1 $\mu$ l
Mix by pipetting, then add:	
Restriction Enzyme,	10u/ $\mu$ l 0.5 $\mu$ l
Final volume	20 $\mu$ l

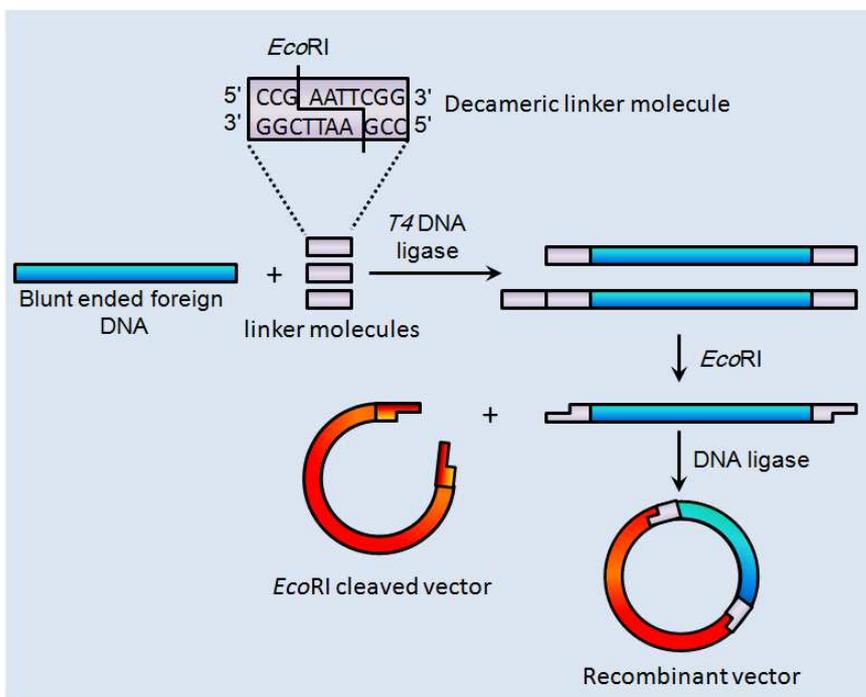
Mix gently by pipetting, close the tube and centrifuge for a few seconds in a microcentrifuge. Incubate at the optimum temperature for 1–4 hours. Add 4 $\mu$ l of 6X loading buffer and proceed to gel analysis. Note that overnight digests are usually unnecessary and may result in degradation of the DNA.

A second method can be used to create recombinant DNA with the DNA fragments and the vectors that lost the sticky ends because of their cutting by a blunt ended restriction enzyme. After cutting the vector (such as the plasmid) and the donor DNA, the polyA can be added, for example, to the 3' end for the plasmid DNA using an enzyme called terminal transferase (figure 6.6). Similarly, polyT is added to 3' end for the donor DNA fragments. Hence, the two fragments can then be hybridized together by virtue of their self-complementary ends and ligated together. If the tails are long enough, the complex can be directly introduced into cells, where the gaps and nicks will be filled and sealed by the cellular enzymes. This procedure is called homopolymer tailing. Although this technique is more difficult than the ligation of the sticky ends, it has an advantage represented by joining of any pair of termini. But there are disadvantages of this that represented by the loss of control on the direction of insertion of joined molecules, add to that, there is no easy way for the recovery of inserted DNA after its insertion into the vector.



**Figure (6.6);** homopolymer tailing. The technique of homotailing by poly-dA and poly-dT tails can be used to construct sticky ends of the desired DNA molecule and to generate recombinant DNA molecule.

The so called linkers can also be used to generate sticky ended DNA molecules that are complementary to each other. Linkers are blunt ended short DNA molecules that have blunt ends containing recognition sequences for a restriction enzyme that generates sticky ends (figure 6.7). The ligation of the linkers with the DNA fragments is very efficient, since the feasibility to obtain high concentration of linkers. After joining of linkers with DNA fragment, the mixture is digested with a restriction enzyme that cuts the linkers and generates the sticky ends. By this way, the blunt ended molecule is converted into sticky ended molecule that can be joined with other DNA molecules. The above account should not be interpreted to mean that gene cloning is an easy technique. In fact, the production and identification of a bacterial cell which has incorporated a functional copy of the desired gene is a formidable task.



**Figure (6.7);** ligation of linkers that contain EcoRI site by T<sub>4</sub> DNA ligase with the blunt ended foreign DNA molecules. Consequently, the resulting DNA molecules have cohesive ends by the restriction enzyme EcoRI. This DNA is joined with the vector that is treated with the same restriction enzyme.

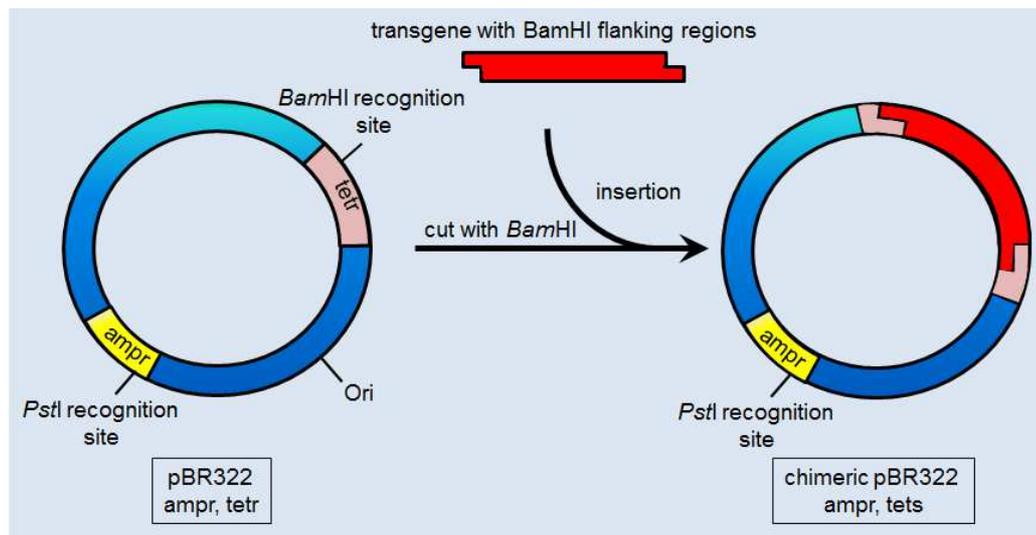
### Third; Tools of DNA Transfer (Cloning Vehicles)

Gene cloning is an indispensable part of any genetic manipulation and involves the joining of a desired DNA fragment to a vector molecule which serves to propagate that DNA segment in bacteria.

Each individual vector characterizes with features which might not be exist in the other vectors. It deserve to be noted that each type of cloning vectors has certain advantages distinguish it from other vectors. However, plasmids are the simplest molecules to deal with. Bacteriophages and other viruses can to be stored properly for long period of times. Cosmids and artificial chromosomes can carry larger DNA fragments for cloning purposes. It's noteworthy that all vectors are shared with multiple general features; they are typically small, the gene transfer from the donor cell into the recipient counterpart is relatively easy, they have known DNA sequences, have at least one origin of replication and they can replicate in the suitable host even when they carry the foreign DNA. Finally, these vectors codes for a phenotype that can be used to identify their presence and the parental vectors (the vectors that don't have foreign DNA) can be identified from the recombinant vectors (the vectors that have the foreign DNA). The most important types of these cloning vectors are:

- a. **Plasmid vectors;** plasmids have many features made them exceptionally advantageous as a cloning vehicles, since they are found in multiple copies within the bacterium, and they can replicate autonomously with respect to the bacterial DNA. The complete sequence of plasmids has been identified. Hence, the locations of the restriction enzymes cleavage sites have been identified as well. Moreover, the plasmids are smaller than the host chromosome. Therefore, they can easily be separated from the later. Add to that, the desired DNA is easily to be isolated by cutting the plasmid with an enzyme specialized to a restriction site at which the original DNA fragment has been inserted. Plasmids were the first cloning vectors. They are easy to isolate and purify, and they can be reintroduced into a bacterium by transformation. Plasmids often bear antibiotic resistance genes, which are used to select their bacterial hosts. A recombinant plasmid containing foreign DNA often is called a chimera, after the Greek mythological monster that had the head of a lion, the tail of a dragon, and the body of a goat. One of the most widely used plasmids is pBR322. This plasmid contains the resistant genes for the both antibiotics, ampicillin and tetracycline and many of restriction sites that exist only once in the plasmid and located within the antibiotic resistance gene (figure 6.8). This arrangement helps in the detection of the recombinant plasmids after performing the transformation. For example, if the foreign DNA is inserted into the ampicillin resistant gene, the plasmid is unable to confer resistance for ampicillin after this insertion. Thus, the transformants that lost resistance to ampicillin contain "chimeric" or recombinant plasmid. As it known, the plasmid DNA don't usually code for any essential function, and the plasmids free cells can divide normally. Plasmids usually exist in

multicopy form, and they can constitute a means to amplify the number of any essential single-copy gene that becomes inserted into their genome. For example, insertion on a plasmid can lead to a rapid amplification of genes needed to achieve high-level of resistance to antibiotics. The significance of what is mentioned above in relation to genetic engineering is that any gene that small plasmids carry is present in large numbers, thereby making it possible that large numbers of the corresponding protein product (of the gene) can be made. Such plasmids thus provide the ideal vehicles to carry the many multiple genes needed for high-level antibiotic resistance, where detoxifying enzymes are required in huge amounts.



**Figure (6.8).** Structure of pBR322 plasmid, and the mechanism of insertion of the gene of interest in it.

Most of the plasmid vectors are multicopy, and one of the earliest plasmid vectors was pBR322, which is one of the most known plasmid vectors since it carries two antibiotic resistance genes, *ampR* and *tetA* that act as markers to select and characterize the recombinants containing these plasmids. In addition to its containing to unique regions for many restriction enzymes that are available to insert DNA fragments. For instance, let us see what is going on when *Pst* I fragment is inserted into *Pst* I restriction site on pBR322 vector, which is located within *ampR* gene. Such insertion will never usually destroy the function of the corresponding gene. Consequently, an active  $\beta$ -lactamase is never further been synthesized. Therefore, the recombinant plasmids can be detected by their ability to confer resistance to tetracycline and not to ampicillin. As for *Bam* HI, the same consequences are met, in such away when the foreign DNA fragment is inserted in *Bam* HI restriction site, the gene that confers resistance to tetracycline for the host bacterium is destroyed. After the transformation of the bacterial host with recombinant plasmid, the bacterium is placed into a growing medium containing ampicillin. Other copy is grown is grown on a medium containing tetracycline to test their

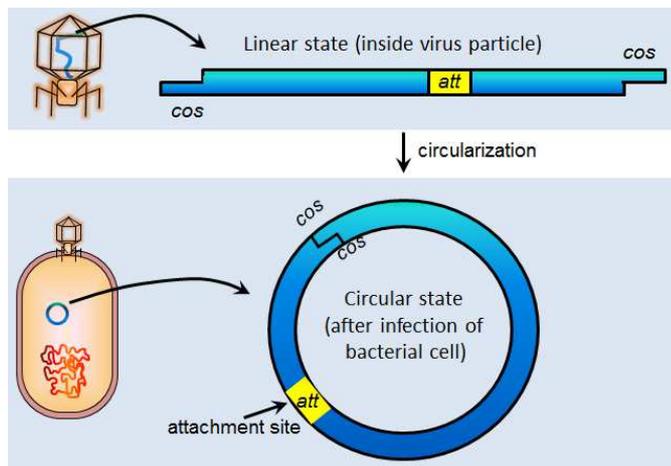
sensitivity to tetracycline. This facilitates the obtaining of the gene of interest through eliminating these bacteria that don't have plasmid at all, or these that contain original plasmid without addition.

- b. **Phage Vectors;** phages have linear DNA molecules, in which the DNA can be inserted through many restriction sites. The recombinant DNA is accumulated after the phage proceeds through its lytic cycle and produces the mature phage particles. The essential advantage for phage transport on the plasmid vehicles is their ability to transfer longer DNA molecules, when the plasmid accommodate DNA fragment. However, when the plasmid vectors accept a 6 – 9 kb DNA fragment, the phage vectors can accept a DNA fragment of about 9 – 20 kb.

Bacteriophage lambda, which infects *E. coli*, has been widely used as a cloning vector. Lambda is a well-characterized virus with both lytic and lysogenic alternatives to its life cycle (see chapter six). Although lambda DNA circularizes for replication and insertion into the *E. coli* chromosome, the DNA inside the phage particle is linear (figure 6.9). At each end are complementary 12 bp long overhangs known as *cos* sequences (cohesive ends). Once inside the *E. coli* host cell, these pair up and the cohesive ends are ligated together by host enzymes forming the circular version of the lambda genome.

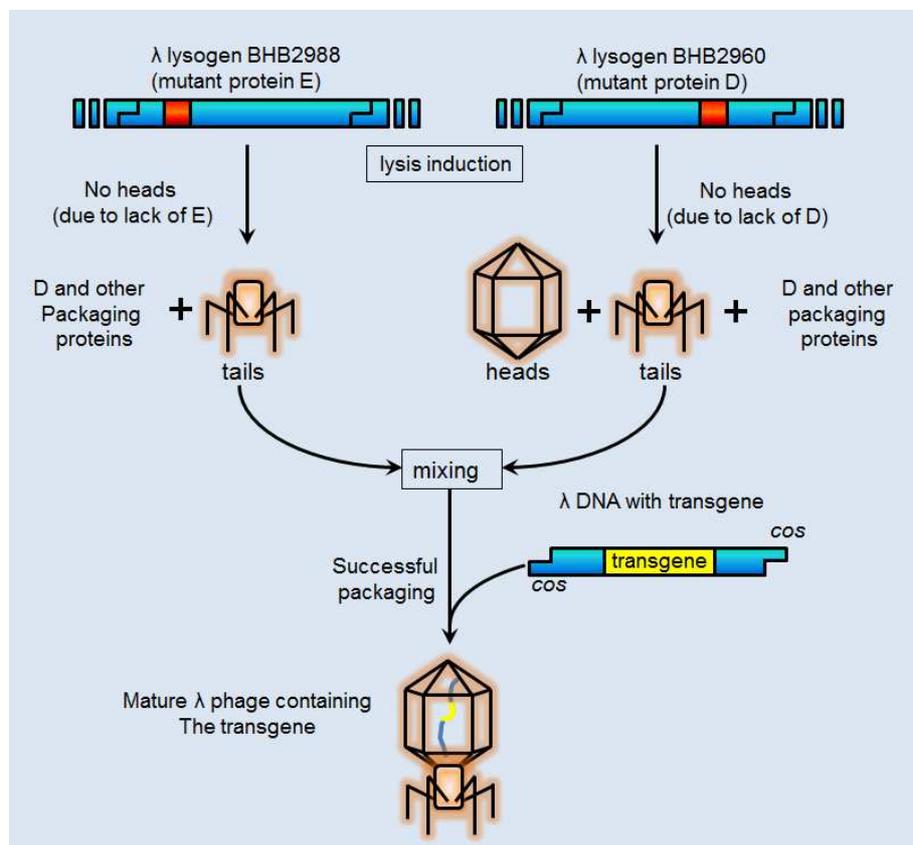
Only DNA molecules of between 37 and 52 kb can be stably packaged into the head of the lambda particle. Small fragments of extra DNA may be inserted into the lambda genome without preventing packaging. However, to accommodate longer inserts it is necessary to remove some of the lambda genome. The left hand region has essential genes for the structural proteins and the right hand region has genes for replication and lysis.

The middle region of the lambda genome is non-essential and may be replaced with approximately 23 kb of foreign DNA. Since the middle region of lambda has the genes for integration and recombination, such lambda replacement vectors cannot integrate into the host chromosome and form lysogens by themselves.



**Figure (6.9);** the genetic material of lambda phage, which is represented by a linear DNA molecule with two *cos* sequences at each end. After the phage injects its DNA into the bacterial host, the DNA circularizes. The two cohesive ends base pair and are ligated together by bacterial enzymes so forming a circle.

The formation of a recombinant molecule between the foreign DNA and lambda phage is much more complex than that taken place in the plasmid vectors. It's not restricted on the integration of the foreign DNA in the middle of the phage to form the linear recombinant DNA of two cohesive ends. Instead, this molecule has to be packaged into the head of the phage efficiently to form a recombinant phage able to infect the bacterial cells. Hence, the *E. coli* cell requires to what is known *in vitro* packaging (figure 6.10). In this technique, a mixture of lambda proteins is mixed with the recombinant lambda DNA *in vitro* to form phage particles. Infecting two separate *E. coli* cultures with two different defective lambda mutants generates the necessary lambda proteins. Each of the two mutants lacks an essential head protein and cannot form particles containing its own DNA. A mixture of the two lysates gives a full set of lambda proteins and when mixed with lambda DNA can generate infectious phage particles capable to infect new *E. coli* cells (figure 6.10).



**Figure (6.10);** In Vitro Packaging of Lambda Replacement Vector A lambda cloning vector containing cloned DNA must be packaged in a phage head before it can infect *E. coli*. Before the DNA can be packaged, the phage head proteins must be isolated. To do this, a culture of *E. coli* is infected with a mutant lambda which lacks the gene for one of the head proteins called E. A different culture of *E. coli* is infected with a different lambda mutant, which lacks phage head protein D. Both *E. coli* cultures are grown with the mutant lambdas and the viruses are induced to enter the lytic cycle. Although the *E. coli* are lysed by the phage, they cannot form complete heads. Instead a soluble mixture of phage proteins is isolated. Each lysate contains phage tails, assembly proteins, and components of the heads, except either D or E. These two lysates are mixed along with the lambda vector containing the cloned DNA. Although mixing is done *in vitro*, the components can self-assemble into a functional phage that can infect *E. coli*.

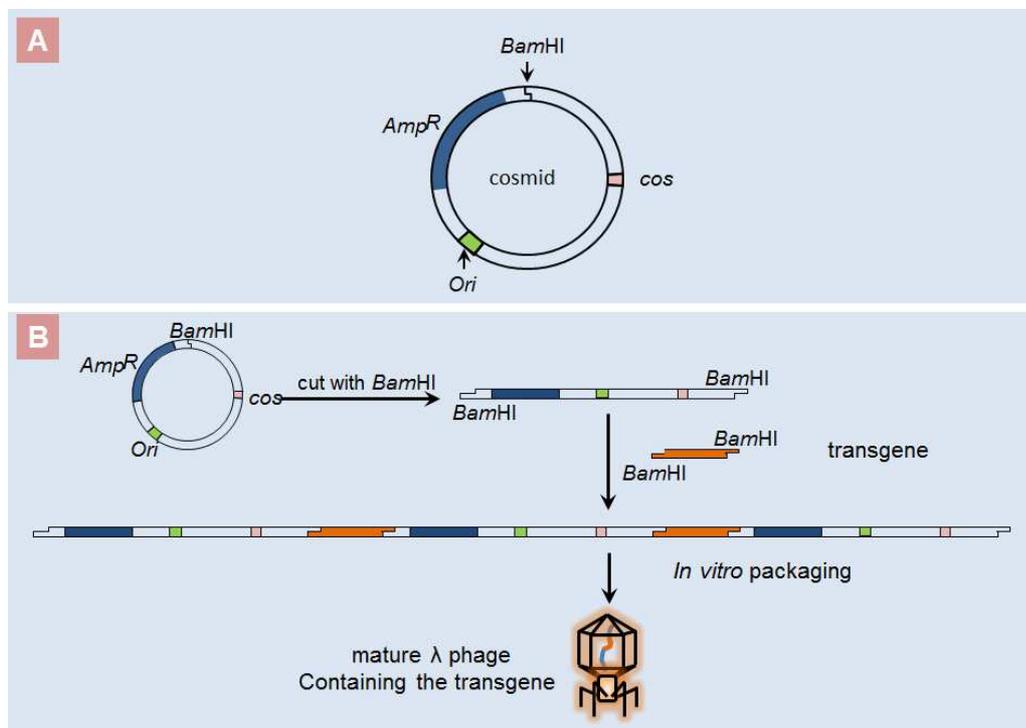
These phages have often been used to generate the genomic libraries. As for the single stranded phages (such as M13), they are used in the determination of DNA sequences (see chapter seven).

- c. **Cosmid Vectors;** Cosmids are small multicopy plasmids that carry *cos* sites that derived from lambda phages and can be packaged into the heads of phages (figure 6.11, A). The lambda genome contains a recognition sequence called a *cos* site (or cohesive end) at each end. When the genome is to be packaged in the head, it is cleaved at one *cos* site and the linear DNA is inserted into the head until the second *cos* site has entered. Thus any DNA inserted between the *cos* sites is packaged. Cosmids typically contain several restriction sites and antibiotic resistance genes.

The only DNA requirements for *in vitro* packaging into  $\lambda$  phage are the presence of two *cos* sites that are separated by 37–51 kb of intervening sequence. Cosmids were developed in light of this observation, and are simply plasmids that contain a  $\lambda$  phage *cos* site. As plasmids, cosmids contain an origin of replication and a selectable marker. Cosmids also possess a unique restriction enzyme recognition site into which DNA fragments can be ligated. After the packaging reaction has occurred, the newly formed  $\lambda$  particles are used to infect *E. coli* cells. The DNA is injected into the bacterium like normal  $\lambda$  DNA and circularizes through complementation of the *cos* ends. The lack of other  $\lambda$  sequences means, however, that  $\lambda$  infection will not proceed beyond this stage. The circularized DNA will, however, be maintained in the *E. coli* cell as a plasmid. Therefore selection of transformants is made on the basis of antibiotic resistance and bacterial colonies (rather than plaques) will form that contain the recombinant cosmid. Since  $\lambda$  phage particles can accept between 37 and 51 kb of DNA, and most cosmids are about 5 kb in size, between 32 and 47 kb of DNA can be cloned into these vectors. This represents considerably more than could be cloned into a  $\lambda$  vector itself. Therefore, using a small cosmid, of say 4 kb, allows inserts of up to about 45 kb to be cloned. Cosmids, like plasmids, are very stable, but the insertion of large DNA fragments can mean that recombinant cosmids are difficult to maintain in a bacterial cell. Repeat DNA sequences are common in eukaryotic DNA, and DNA rearrangements can occur *via* recombination of the repeats present on the DNA inserted into the cosmid. The major difficulty in working with cosmids is, however, the production of linear, ligated DNA fragments in which the cosmid and insert are concatamerized together (figure 6.11, B).

Phage or cosmid vectors are used in the construction of genomic libraries. Usually, the DNA library is a group of cloned DNA fragments

in the cloning vector that can be used to search the DNA of interest. There are two types of these libraries are used to obtain the DNA of interest. The first type is called genomic DNA libraries. This library is made from the genome of the organism of interest. For example, the genomic mouse library is made by the partial digestion of the nuclear mouse DNA by restriction enzyme to produce large number of different DNA, but all of which have homologous cohesive ends (see below). Then, the DNA fragments are ligated by phages that derived from lambda phages or by cosmid vectors that are ligated by the same enzyme. This library contains all the mouse nuclear sequences and any desired gene in the mouse can be looked for. Its noteworthy to say to not each clone contains a whole gene since – in many cases – restriction enzymes make their cuts within the gene. The second type of DNA libraries is called cDNA libraries. The cDNA library can be made by using reverse transcriptase that is derived from retroviruses (see below).



**Figure (6.11, A and B);** DNA cloning in cosmid vector. To clone large pieces of DNA into cosmid vectors, both must have compatible sticky ends. The cosmid vector is first linearized so that each end has a *cos* site. Then the linear cosmid is cut with *Bam*HI, which generates sticky ends with the overhang sequence GATC. The genomic DNA from the source of interest is also digested. Instead of *Bam*HI, this DNA is partially digested with *Mbo*I, which also generates a GATC overhang. Partial digestion leaves some sites uncut and allows large segments of a genome to be isolated. These segments are mixed with the two halves of the cosmid and joined using ligase. The final constructs are packaged into lambda particles *in vitro* and are used to infect *E. coli*.

- d. **Artificial Chromosomes;** In addition to the three major types of vectors, which are plasmids, bacteriophages, and cosmids, artificial chromosomes may be considered as an important type for cloning vectors. The artificial chromosomes are common tools in gene transfer since they contain large

quantities of genetic material. Yeast artificial chromosome or YAC is one of the widest artificial chromosomes used. The YACs are DNA extensions contain all the necessary elements to spread chromosomes in the yeasts, which are the origin of replication, centromere, and telomeres. They also have sites for restriction enzymes and genetic markers where they can be tracked and selected. The YAC is cleaved by suitable restriction enzyme, which cleaves it and allows a fragment of foreign DNA to be inserted between the centromere and telomere. Thus, the YACs contain DNA fragments between 100kb into 200kb in size, and can be place in *Saccharomyces cerevisiae*, and can be replicated side by side with the real chromosomes.

The bacterial artificial chromosomes are alternative cloning vehicles, their utilization is increasing proportionally. The BAC are cloning vehicles based on F plasmids of *E. coli* (see chapter nine; F factors), and contain suitable sites for restriction enzymes and selectable marker, such as the resistance for chloroform. The modified plasmid is cleaved in the restriction site, and the foreign DNA fragment is ligated that estimated to be more than 300kb using the DNA ligase. The BACs proliferate in *E. coli* after its entry by the electrical pulses or electroporator (see below). It's possible for this vehicle to be reproduced and modified easily, and doesn't suffer from any recombination as it's noticed in YACs. The recombination being don't occurring has an advantage that held in the fact that the integrated DNA cant usually be rearranged in such away it keep it's own sequence without being changed. Since the BACs vectors can carry large fragments of DNA, the artificial chromosomes are particularly important in the identifying the genomic sequences.

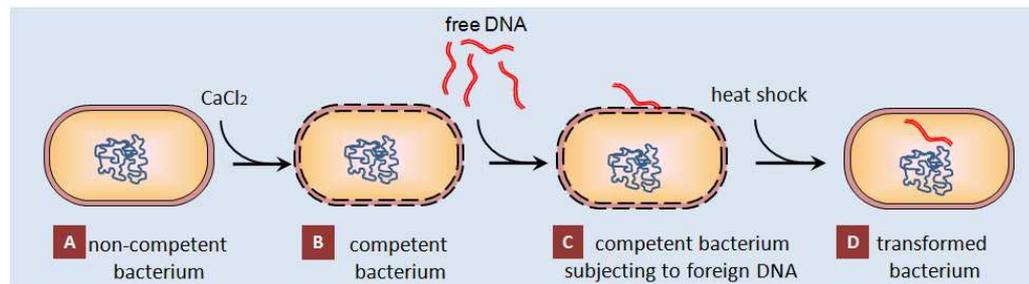
#### **Fourth; Tools of Foreign Genes Entry into the Cells**

After constructing the recombinant DNA molecule, which usually consists of the DNA of interest inserted into a suitable cloning vehicle, it must introduce this vector into a suitable host to produce large quantities of it. This is done in order to be studied by the genetic engineers. Generally, the hosts are divided into two major types; prokaryotic hosts and eukaryotic hosts.

##### **a. Genes entry into prokaryotic cells**

Actually, it's possible to introduce both prokaryotic and eukaryotic genes in bacteria, but the way that is used in each of them differs from each other. In prokaryotes, such as bacteria, after isolating the desired DNA fragment, its ligation into the vector by suitable restriction enzyme and its forming the recombinant transfer molecule, this molecule is introduced into a suitable host usually by transformation. The actively growing cells for the bacteria of interest, such as *E. coli*, in diluted solution of calcium chloride ( $\text{CaCl}_2$ ) in an ice surrounded medium,

which relatively increases the ability of bacterial cells to uptake foreign DNA (figure 6.12). The exposure to  $\text{CaCl}_2$  is usually for 30 min. the incubation of this medium for a short period allow cells to uptake the foreign DNA and to form the transformants.



**Figure (6.12);** the chemical transformation of bacterial cells. The treatment of cells with calcium ions can make these cells competent to uptake DNA. The DNA may be adhere on the surface of the bacterial cell, and by a heat shock, the entry of foreign DNA is taken place.

### ***E. coli* transformation protocol**

There are several competent cells that are commercially available for transformation purposes such as; BL21(DE3) pLysS, BMH 71-18 *mutS*, HB101, and JM109. Each strain has its own characteristics.

#### **Standard Transformation Protocol (Promega – USA)**

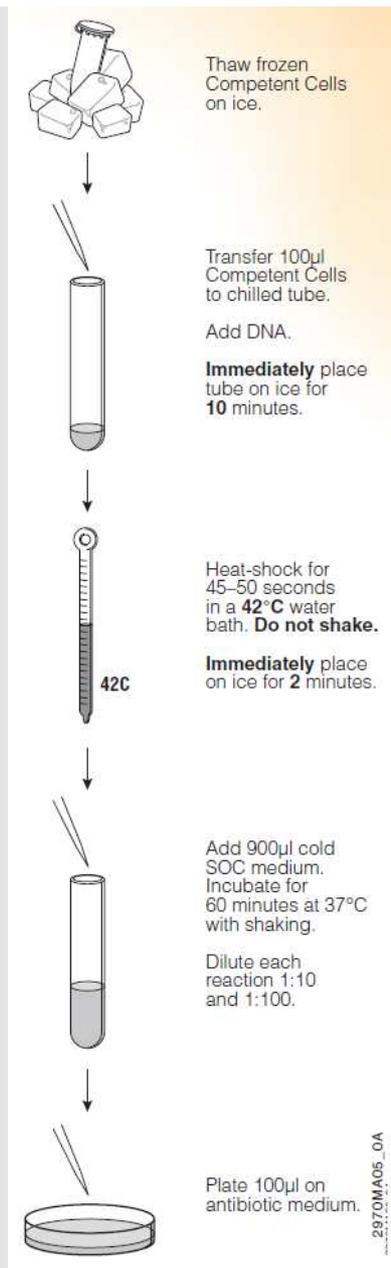
##### **Materials to Be Supplied by the User**

- LB or SOC medium

**(LB medium with ampicillin;** , mix 10g/L Bacto®-tryptone, 5g/L Bacto®-yeast extract, and 5g/L NaCl, Adjust the pH to 7.5 with NaOH., Autoclave to sterilize. Allow the autoclaved medium to cool to 55°C, and add ampicillin (final concentration 100µg/ml). For LB plates, include 15g agar prior to autoclaving.)

- LB plates with antibiotic
- 17 × 100mm polypropylene culture tubes, sterile
- IPTG (IPTG stock solution, 0.1M; 1.2g IPTG , Add water to 50ml final volume. Filter-sterilize through a 0.2µm filter unit, and store at 4°C.)
- X-Gal (Available from Promega (Cat.# V3941) at a concentration of 50mg/ml in dimethylformamide.)

1. Chill sterile 17 × 100mm polypropylene culture tubes on ice, one per transformation (e.g., Falcon™ 2059). Use of a standard microcentrifuge tube reduces the transformation efficiency by approximately 50% due to inefficient heat-shock treatment of the cells.
2. Remove frozen Competent Cells from –70°C, and place on ice for 5 minutes or until just thawed. Once the cells have thawed, pipet quickly or use chilled (4°C) pipette tips to prevent the cells from warming above 4°C (figure 6.13).
3. Gently mix the thawed Competent Cells by flicking the tube, and transfer 100µl to each chilled culture tube.



**Figure (6.13).** Standard Transformation Protocol from Promega, which consists of the following steps;

1. Chill sterile 17  $\times$  100mm polypropylene culture tubes on ice.
  2. Thaw frozen Competent Cells on ice until just thawed.
  3. Gently mix the thawed Competent Cells by flicking the tube. Transfer 100 $\mu$ l to each chilled culture tube.
  4. Add 1–50ng of DNA or 1 $\mu$ l (0.1ng) Competent Cells Control DNA per 100 $\mu$ l of Competent Cells. Quickly flick the tube several times. (Use the Competent Cells Control DNA to determine transformation efficiency.)
  5. Immediately return the tubes to ice for 10 minutes.
  6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. Do not shake.
  7. Immediately place the tubes on ice for 2 minutes.
  8. Add 900 $\mu$ l of cold (4°C) SOC medium to each transformation reaction. Incubate for 60 minutes at 37°C with shaking.
  9. For each transformation reaction, dilute cells 1:10 and 1:100. Plate 100 $\mu$ l of the undiluted cells and 1:10 and 1:100 dilutions on antibiotic plates. Incubate the plates at 37°C for 12–14 hours.
- For the control, dilute the cells 1:10. Plate 100 $\mu$ l (0.001ng) on LB/ampicillin plates. If using BL21(DE3)pLysS Competent Cells, do not dilute; spread 100 $\mu$ l of these cells directly onto antibiotic plates.

4. Add 1–50ng of DNA (in a volume not greater than 10 $\mu$ l) per 100 $\mu$ l of Competent Cells. Move the pipette tip through the cells while dispensing. Quickly flick the tube several times.  
**Note:** To determine the transformation efficiency, we recommend using 1 $\mu$ l (0.1ng) of Competent Cells Control DNA at this step.
5. Immediately return the tubes to ice for 10 minutes.
6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C.  
**Do not shake.**
7. Immediately place the tubes on ice for 2 minutes.
8. Add 900 $\mu$ l of cold (4°C) SOC medium to each transformation reaction, and incubate for 60 minutes at 37°C with shaking (approximately 225rpm).  
**Note:** Use high-quality deionized water (e.g., Milli-Q® or NANOpure®) for SOC medium (see recipe in Section 5). If LB or other medium is used, transformation efficiencies will be reduced.
9. For each transformation reaction, we recommend diluting the cells 1:10 and 1:100 and plating 100 $\mu$ l of undiluted cells and 1:10 and 1:100 dilutions on antibiotic plates (see Notes

1–3). Incubate the plates at 37°C for 12–14 hours.

After the entry of the recombinant vector molecules into the recipient cells, we must refer to the fact that small ratios of the cells are transformed, as for others, they fail in uptaking the foreign DNA (the new plasmid). Hence, the necessity to the presence of some ways to enable us to select the truly transformed cells is emerged. Therefore, the vectors (such as plasmids) that are used in DNA inserting must contain some selectable markers, such as the resistance for some antibiotics.

After isolating the gene of interest, its ligation into the suitable cloning vector, and its entry into the host cell by certain way, a final step remains, which is the preparation of the suitable circumstances for this gene to express about itself, and to produce the desired protein. It must refer that the cloned gene is not usually expressed in the host cell without the presence of certain modifications ensure the expression of this gene in the host cell. To be expressed, the foreign gene that is inserted in the recombinant molecule must contain promoter that must be recognized by the host RNA polymerase. The translation of the mRNA copy depends on the presence of leader sequences, and on the suitable mRNA modifications to ensure its binding with ribosomes. These diverse processes in prokaryotes are widely different from these present in eukaryotes. When the foreign DNA is introduced in prokaryotic cells, such as bacteria, it must be supplied with the leader sequence in order to assist it in protein synthesis in bacteria. Eventually, introns must be removed from eukaryotic genes since the prokaryotic host doesn't excise the introns after mRNA transcription. Consequently, the eukaryotic protein cannot be active without removing its introns before translation.

The problems of foreign genes expression have been largely conquered in the host cells by the help of special vectors called expression vectors. Usually, these vectors are derived from pBR322, and contain the necessary transcription and translation signals. They have useful restriction sites for these sequences, where the foreign DNA can insert in. Some expression vectors contain portions of *lac* operon, which can efficiently regulate the expression of the cloned genes in the same mode of operon. As for to the entry of eukaryotic genes into bacteria, since the beginning of genetic engineering, many scientists were excited in this respect particularly after the success of some prokaryotic genes to express about themselves in the bacterial cells. After the discovery of the universality of the genetic code, it became known that the genetic code gives the same amino acid whether in prokaryotes or eukaryotes. They tried, for this reason, to introduce the eukaryotic genes in bacteria in the same way used in the introduction of prokaryotic genes. These trials

were taken place for the hope to succeed these genes to express about themselves in bacteria, but nothing was obtained. These failure trials were continued until the fact of introns was discovered in the eukaryotic genes. Since, there is no system in bacteria that can remove introns and connect exons together, therefore, a tool that enable the researchers to remove the unwanted introns before the entry of eukaryotic genes into bacterial host must be found.

In eukaryotic cells, many genes are transcribed into only mRNA in specialized cellular types. For example, the mRNA molecules that code for globin proteins are exist in the reticulocytes only. Similarly, the albumin coding mRNA, produces the essential protein in the serum, is only produced from the liver cells during albumin synthesis. The specialized DNA sequences that code for mRNA in the particular cell can be cloned by synthesis of DNA copies for the isolated mRNA from that type of cells, then, these DNA copies are inserted in the plasmid vectors, or in the other suitable cloning vectors.

The DNA copies of mRNA molecules are called complementary DNA (cDNA). In addition to its representation for the sequences that code for mRNA in a particular cell type, the cDNA losses the noncoding introns that present in the DNA genomic sequences. Thus, the amino acid sequences of the protein can directly be determined from the nucleotides sequence for its corresponding cDNA. Many of genes of higher organisms are considered very large and they cannot be inserted into plasmids and even in lambda phages, because of their long introns that intervening the exons of these genes. This in turn, made the researchers in this field to construct cDNA for the genes to be cloned.

#### **b. Genes entry into eukaryotic cells**

The researchers in the field of genetic engineering were devoted all of their every effort in order to develop the techniques for genes entry into the eukaryotic cells. All of these trials were performed because of what of this process have in applicable importance, where these cells of higher organisms enjoy with a unique sort of processing that could not be provided in the prokaryotic cells. Many of these techniques were used successfully in transforming many of the prokaryotic cells. These require to special treatments each of its type. For example, some of yeasts, fungi, and plant cells have cell walls that are required to be digested to produce the protoplast (cell that has had its cell wall removed) before the DNA is being uptake, while the animal cells don't contain any thick cell wall to surround them. This in turn, facilitates the entry of genes form this aspect. Hence, the variability of treating these multiple origin eukaryotic cells is emerged according to their physical

nature. There are many methods to introduce the foreign DNA into the eukaryotic cells; the most common are summarized by the following:

1. **DNA uptake (co-precipitation) technique;** the general method to introduce the foreign DNA into the mammalian cells involves co-precipitation of the DNA with calcium phosphate, then, the mixture is presented to the cells of the cultured medium (figure 6.14). The individual DNA usually inserts as multiply copies in the cellular genome.

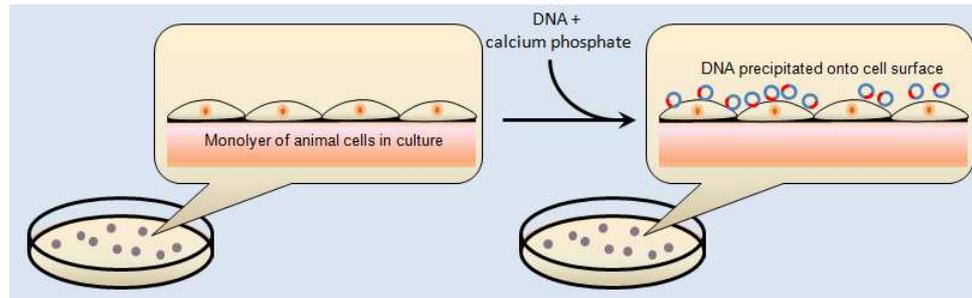


Figure (6.14); the strategy of foreign DNA entry through its precipitation on to the animal cells.

2. **Electroporation technique;** to increase the efficiency of DNA uptake, the electroporation is frequently used. This procedure is usually applied in yeasts, fungi and in plant cells, and less frequently in animal cells. In this procedure, the cells are subjected to a brief electrical pulse by a pulser device, which causes a localized transient disorganization and breakdown of the cell wall, making it permeable for diffusion of recombinant DNA molecules (figure 10.15).

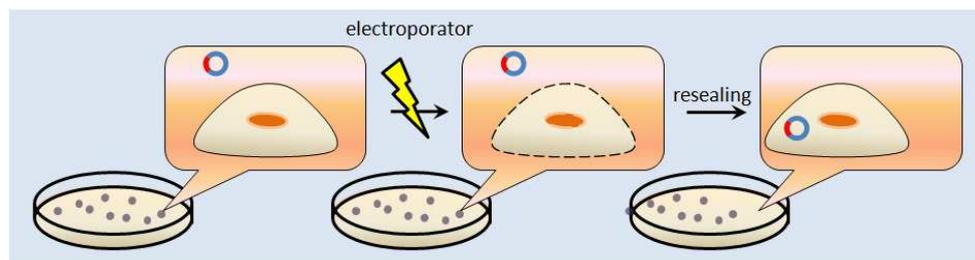


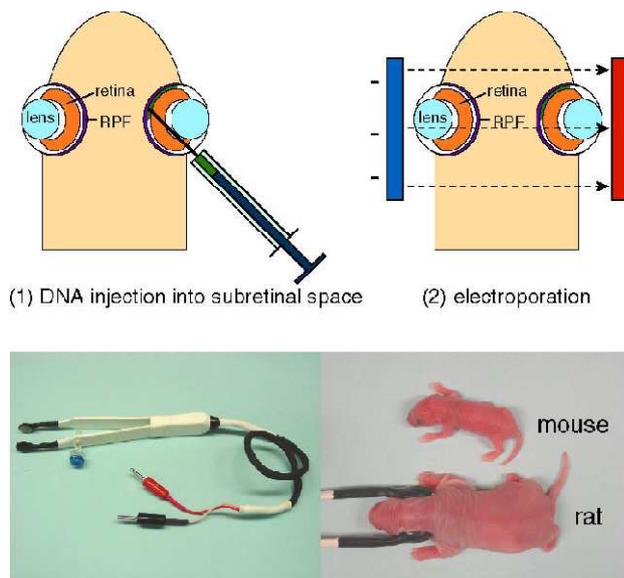
Figure (10.15); electroporation technique. The electrical pulser applies calculated electrical pulses on the foreign DNA subjected cells. This leads to the formation of transient pores in the cellular membranes. This may lead to the entry of this DNA into the cells to form the recombinant cells.

There are many "noticeable" advantages of this physical procedure perhaps one of the most prominent advantages is its high velocity, its low cost compared with other microinjection techniques. In addition to its ability to produce high percentage of genetically engineered cells. Transformation efficiencies much higher than those obtained by chemical methods can be achieved by electroporation. A pulse is easily delivered by choosing a preset program and touching a single button (figure 6.16).



**Figure (6.16).** MicroPulsar Electroporator from BIO-RAD (Cat # 165-2100), which has the following specifications (input voltage; in-line switching, 100 – 120V or 220 – 240V, input current; 2 amp RMS (100-120V), 1 amp RMS (220-240V), maximum output voltage and current; 3000V peak, output waveform; decaying or truncated exponential waveform with RC time constant of 5.0 ms assuming loads of 3.3kΩ, operating environment; temperature 0-35°C, dimensions; 31x21x8cm (LxWxH), weight; 2.9kg.

Despite of its ability, these high power electrical fields have harmful physical effects on the survival of these subjected cells (figure 6.17). Therefore, the researchers want to optimize the conditions of this technique as much as possible to avoid the damage of the cells that are intended to be genetically engineered.



**Figure (6.17).** Protocol for *in vivo* electroporation into mouse/rat retina. This experiment requires several equipments; Square pulse electroporator CUY21 (Nepagene, Japan), Tweezertype electrodes (BTX, model 520, 7mm diameter), Model 522 (10mm diameter) works as well, Disposable 30G1/2 needle (Becton Dickinson #5106), Cotton swab, Hamilton injection syringe with a 33G blunt end needle (#0159666), or Hamilton injection syringe with a 32G blunt end needle (#87931).

- 3. Protoplast generation technique;** it's performed in the wall surrounded cells. In which, the walls of these cells are removed by the addition of number of available enzymes to generate what is called protoplast in the optimum conditions (figure 6.18). After this stage, the foreign DNA is added into protoplast. Then, the protoplast is regenerated into its normal state when the suitable conditions are available. This method suffers from the difficulty of reforming protoplast into the normal cellular state.

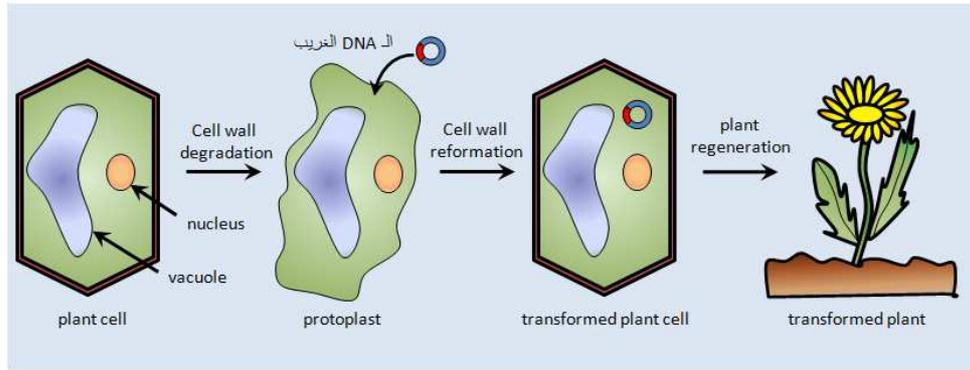


Figure (6.18). The entry of foreign DNA into the protoplast cells.

4. ***Agrobacterium* injection technique**; it which Ti plasmids are used for plant cells. One of the most used vectors in genetic engineering in plants is Ti plasmid, which means "tumor inducing plasmids" that exists in *Agrobacterium tumefaciens*. In dicotyledons, this plasmid produces tumor cells known as crown galls. Its possible for this plasmid to be altered, where it can carry a passenger DNA into plants without transforming cells into tumor. Within Ti plasmid, there are genes in a fragment called T DNA, which means "transfer DNA". This region contains the genes that code for the necessary enzymes for abnormal amino acids synthesis, opines, and other materials that induce abnormal cellular growth (*i.e.* tumor) (figure 6.19).

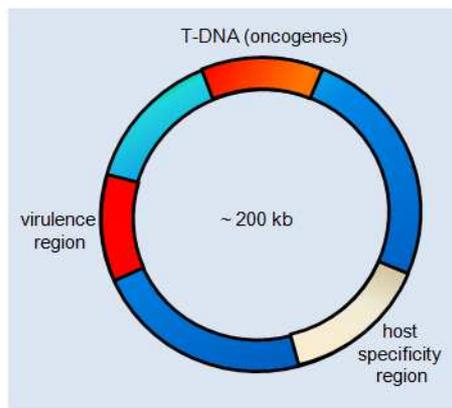
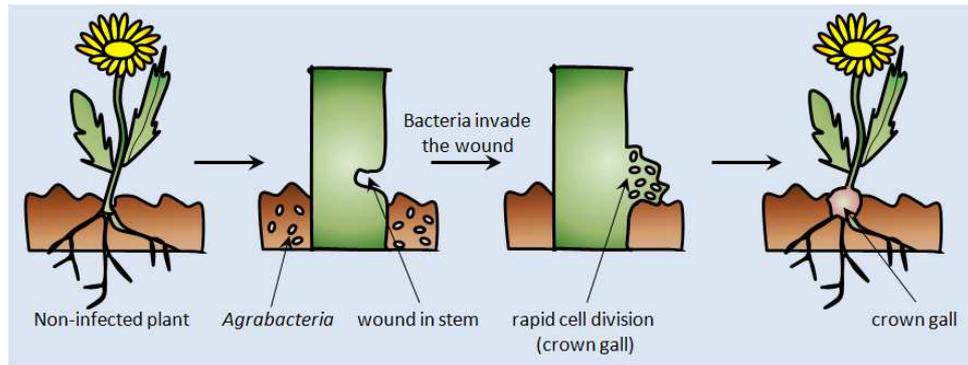


Figure (6.19). The structure of the normal Ti plasmid that exists in *Agrobacterium tumefaciens*.

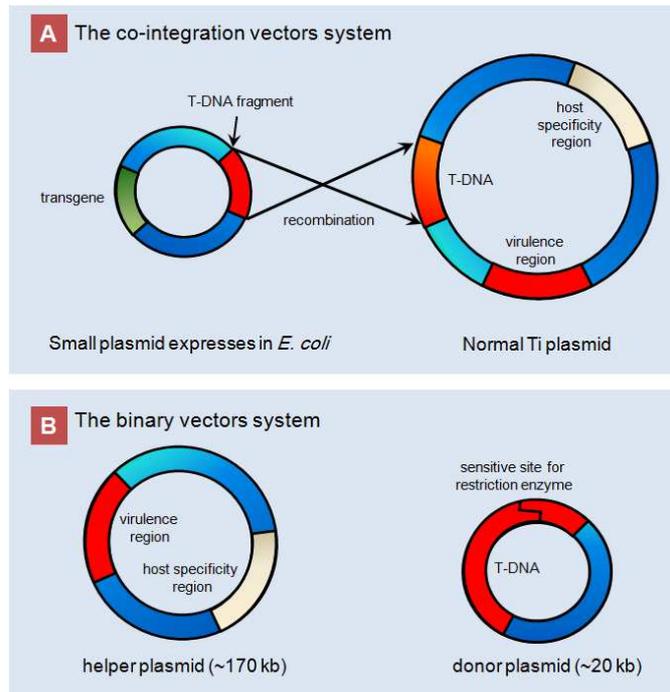
This plasmid has been isolated from *Agrobacterium tumefaciens*, which is a bacterium that grows in the soil and infects plants to cause a tumorigenic disease called crown galls disease (figure 6.20). in the case of infection, a small fragment (about 20kb) is transferred that is called T DNA, which is exist in Ti plasmid, and integrates on the plant chromosome. The transfer process is controlled by *vir* (virulence) gene that is located in Ti plasmid.



**Figure (6.20).** The method of plant cells infection by *Agrobacterium tumefaciens* and the correlation of this with the inflection of crown gall disease.

The size of Ti plasmid makes it not suitable to act as cloning vector. This is at least because of two reasons; (1) when plant cells infected with Ti plasmid, they converted into tumor cells that cannot be converted into normal plants. (2) The size of Ti plasmid is 150 – 200 kb make it so difficult to be genetically manipulated. Therefore, two strategies were developed to clone the genes by this plant vectors. The first strategy is known as co-integration vectors, while the second strategy is known as binary vectors. In the first one, the new DNA integration on Ti plasmid is produced from the recombination of small plasmid vector, such as *E. coli* vectors, with Ti plasmid of *Agrobacterium*. The recombination takes place between two homologous regions that localized in both plasmids (figure 6.21, A).

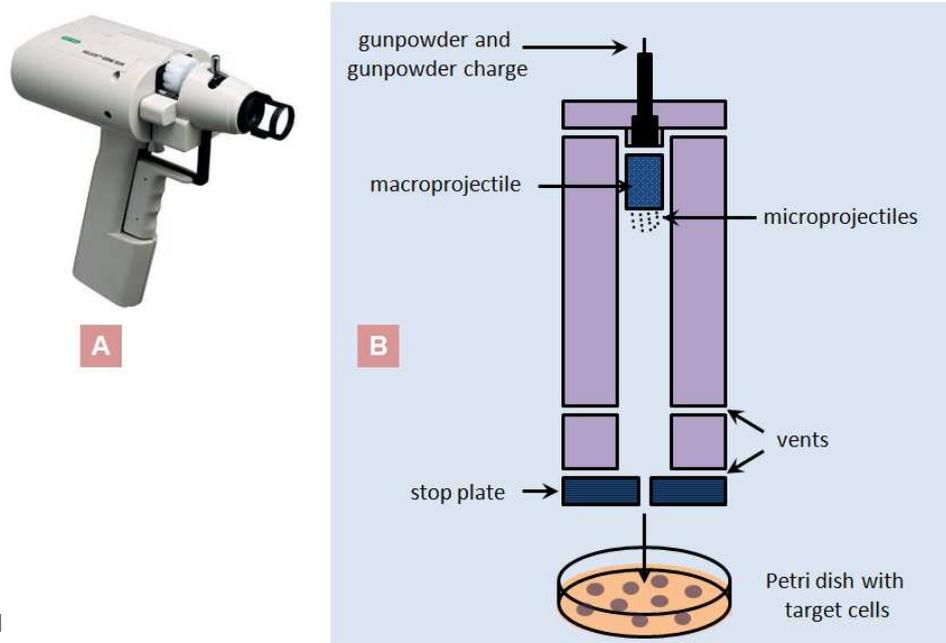
In the second strategy, a binary vector system is designed, which consists of helper plasmid and donor plasmid. The helper plasmid is a Ti plasmid, but in a disarmed form because of the deletion of T DNA fragment (tumorigenic genes carrier) entirely. Donor plasmid is a plasmid expresses in *E. coli*, and carries a brief fragment of T DNA. This means, that the two plasmids work together in a complementary way to each other, in such away the donor plasmid carries the integrated gene surrounded with the terminal sequences of T DNA that are specialized in excision, whereas helper plasmid provides the enzymes (that coded by *vir* genes) that are necessary to direct the transfer of the recombinant T DNA (that carried the inserted gene to be cloned). This is applied practically by carrying the *Agrobacterium* strain the disarmed helper plasmid, while the donor plasmid is injected in the bacterium, this leads to the formation of the binary cloning system (figure 6.21, B).



**Figure (6.21);** cloning strategy using cloning vehicles that are derived from Ti plasmid. (A) co-integration vectors system, in which the small plasmid carry the gene to be cloned directly, and the gene is transferred by recombination with the normal Ti plasmid. (B) binary cloning system; the donor and helper plasmids complement each other when present together in the same *Agrobacterium tumefaciens* cell. The T DNA carried by donor plasmid is transferred into chromosomal DNA by proteins coded by genes carried by helper plasmid.

- 5. Biolistic technique;** the name of this technique is came from the word "biological ballistic". The gene gun is a device which "literally" shoots recombinant DNA into the target cells. This technique is a direct physical method to genetically modify cells, in which a thin coat of DNA is deposited onto the surface of 0.5 -1.5  $\mu\text{m}$  of tungston or gold microbeads. The DNA-coated beads are then loaded and fired from a gene gun by explosive, electric, or pressure charge. The DNA-coated beads are bombarded onto plant tissues, enter the cells, and are integrated into the cell chromosomal DNA randomly (figure 6.22, A and B).

The gene gun is used particularly in infecting cells that are difficult to be infected with nucleic acids in other methods such as plant cells. Although this technique has been originally developed for pant cells, it can be applied in animal cells, tissues, organs, yeasts, and even in other bacterial and microbial organisms.



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**Figure (6.22)**; the gene gun (biological ballistic firing device) that used to fire the coated DNA beads into plant cells. (a) Gene gun device (Bio-Rad corporation – USA), (b) an illustration shows the mode of biological ballistic firing by this device. The DNA is coated onto microprojectiles, which are accelerated by the macroprojectile on firing the gun. At the stop plate the macroprojectile is retained in the chamber and the microprojectiles carry on to the target tissue.

6. **Pronuclear microinjection technique**; this technique was regarded as one of the most effective method by which some transgenic animals can be generated. This technique is done by injecting male pronucleus of fertilized oocyte using a specialized micropipette that has been with certain devices to perform these tasks (figure 6.23).

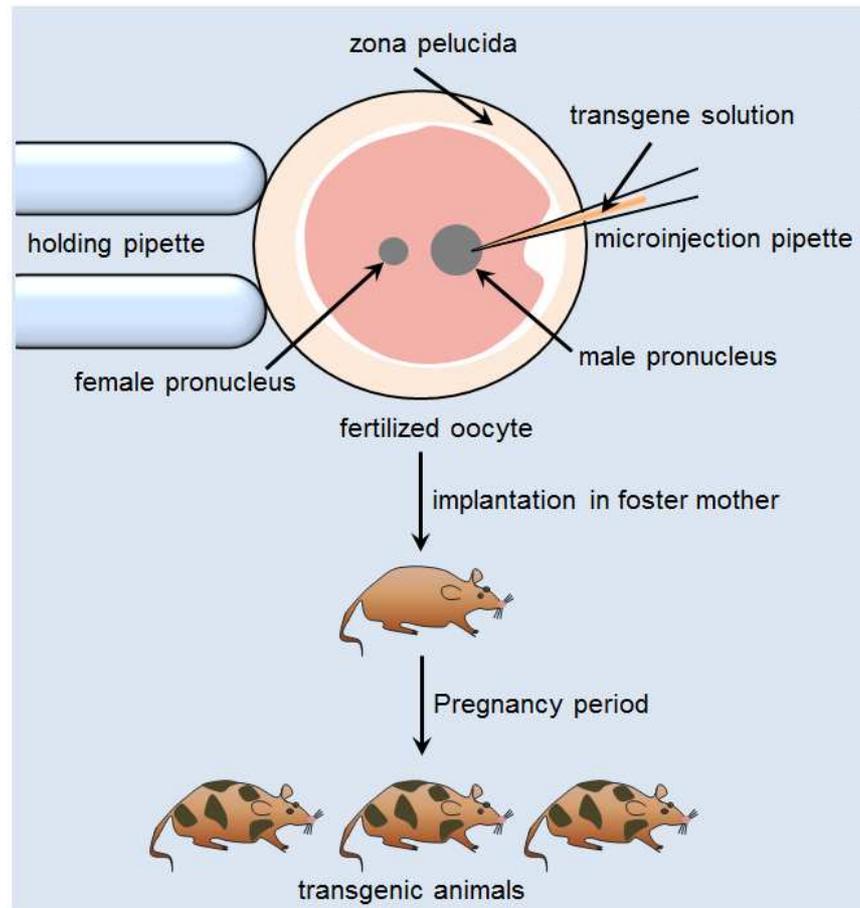
The reason for injection of the transgene into the male pronucleus attributed to the fact that male pronucleus is larger than female counterpart. Off course, this facilitates the mission of the researcher who intends to inject the gene properly. For expression purposes, the gene of interest must be properly constructed with a promoter region and other control elements to direct tissue-specific production of the protein. The transformed zygote is implanted into a surrogate mother to give birth to transgenic offspring (figure 6.23).



**Figure (6.23).** Micromanipulator (TransferMan® NK 2; a Workstation for ICSI or ES cell transfer) from Eppendorf – Germany, which has the following components; (2 x TransferMan NK 2 micromanipulators with CellTram Air and CellTram vario microinjectors, shown with Olympus® IX 71 microscope).

This technique is the most direct way in this field to generate transgenic animal directly as in zygotes and sometimes the foreign DNA is stably integrated into the host genome to generate the transgenic animal. Very fine glass micropipette is used in this type of transfer, through which the transgene is injected into the newly fertilized cell that stabilized on by a holding pipette (figure 6.24).

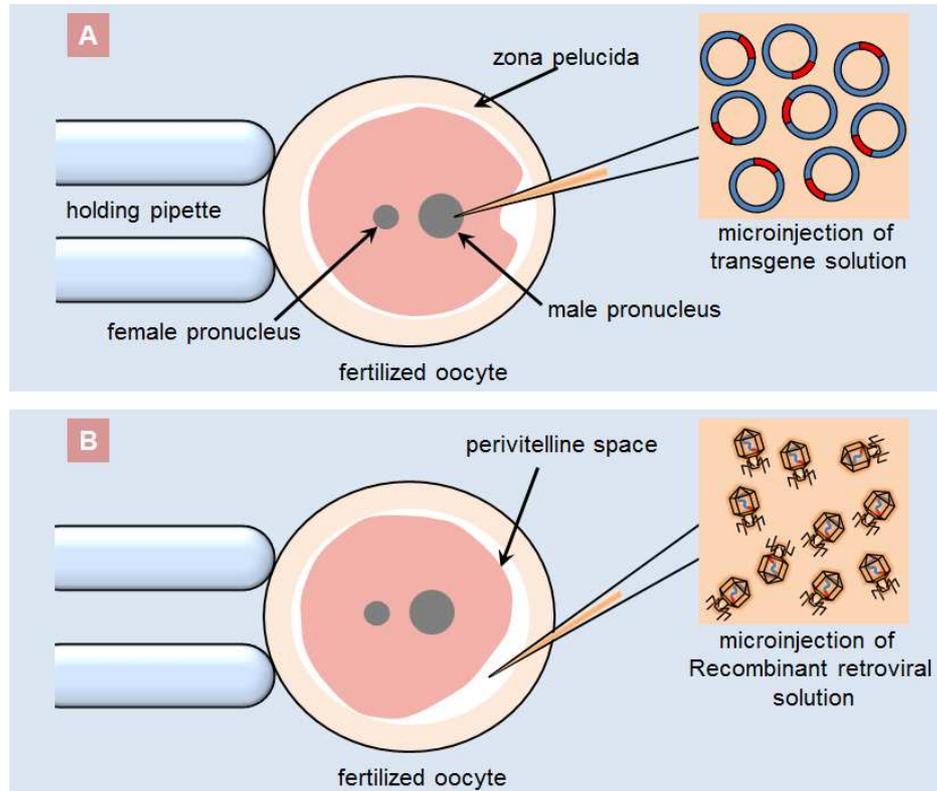
It must be noted that this technique is restricted on the vertical gene transfer exclusively. The meaning of vertical gene transfer is the transfer of the gene from one generation to other one (from parents to offspring). This doesn't occur except by manipulating germ cells. When its succeed, a new born transgenic animals. Since this technique specialized in the injection of the foreign gene into male pronucleus of oocytes, which off course are germ cells, it cannot be used in horizontal gene transfer, i.e., gene transfer through somatic cells that occurs either *in vitro* such as growing cells in Petri dish, or by injecting gene *in vivo* for gene therapeutic purposes, such as injection of viral coated gene, or injection of liposome mixed gene (see below).



**Figure (6.24).** Illustration of pronuclear microinjection. the desired DNA is pipetted into the pronucleus by the micropipette that used to pierce oocyte. Following withdrawal of the micropipette, surviving oocytes are reimplanted into the oviducts of pseudopregnant foster females to may generate new born genetically engineered babies.

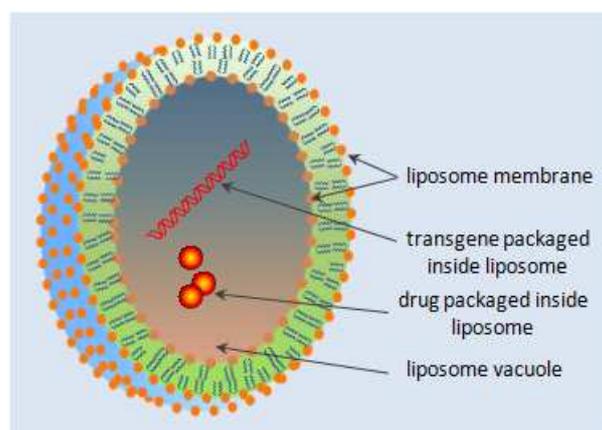
7. **Viral infection (transfection) technique;** Viral-mediated transfer provides a convenient and efficient means of introducing eukaryotic genes into mammalian cells. This method involves the use of a number of viruses, such as simian virus 40 (SV40), bovine papilloma virus (BPV), Epstein-Barr virus (EBV), and retrovirus. Baculovirus is also included, although insect cells are used as the host in this system. The mammalian expression vectors for this purpose are derived from the regulatory sequence (promoter and enhancer) that belongs to viruses. Using this technique, the gene can be injected vertically (figure 6.25), or horizontally in the somatic cells directly for gene therapeutic purposes as it's shown above. May be the most important problems that correlated with the gene transfer by this way are represented by the biohazard emerged from the practical dealing with these viruses. Although many researchers were did there every efforts to delete the hazardous biological genes, such as viral oncogenes, form these viral vectors, but the potential biological danger on the health of researchers and recipients for these therapeutics still exist. This

needs to pursue the development of nonviral gene delivery techniques such as liposomes.



**Figure (6.25);** comparison between pronuclear microinjection (A) and retroviral mediated gene transfer (B), the retroviral particles have been injected in the perivitelline space that is located between zona pellucida ,the embryo outer protection membrane that prevent the entry of recombinant viruses, and the oocyte membrane.

8. **Liposome mediated DNA transfer technique;** it's a pure chemical technique for gene transfer that depends on DNA binding with liposome. It can be used – as in viral vectors – in horizontal gene transfer. Liposomes are hollow spheres surrounded with membranes made up of fatlike molecules called phospholipids (figure 6.26).



**Figure (6.26);** Liposomes can carry DNA genes. Like eukaryotic cells, liposomes are surrounded by phospholipid bilayer membranes. Liposomes carrying therapeutic DNA genes or drugs can fuse with the plasma membrane of a eukaryotic cell to deliver the contents inside the cell.

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oday liposomes are an important part of biological, pharmaceutical, and medical research. Because liposomes are the most effective carriers for the introduction of many different types of agents into cells, the applications of liposome-based samples and products are extremely wide. Liposomes can be synthesized by special devices prepared from Hoefer (figure 6.27).



**Figure (6.27).** Liposome construction device (Liposomat), from Hoefer – USA, cat # SP-746400, which has the following specifications (Liposomat Device for Preparation of Liposomes 3 ml to 50 ml with Dual Pump, Flow-Through Dialysis Chamber and 100 Membranes –MWCO 5,000 Daltons, qty/1). The Liposomat is ideal for the preparation of liposomes of volumes from 3 ml to 50 ml or higher, The system has two serpentine channels superimposed on each other and separated by a membrane, Each channel has a volume of 3 ml and a length of 3 meters. The mixed lipid/detergent micelles run through one of the channels while the buffer flows through the other channel, Due to controlled dialysis and the high surface area in the system, liposomes can be formed within 30 minutes, The serpentine chambers can also be immersed in a water bath for liposome production at constant temperature.

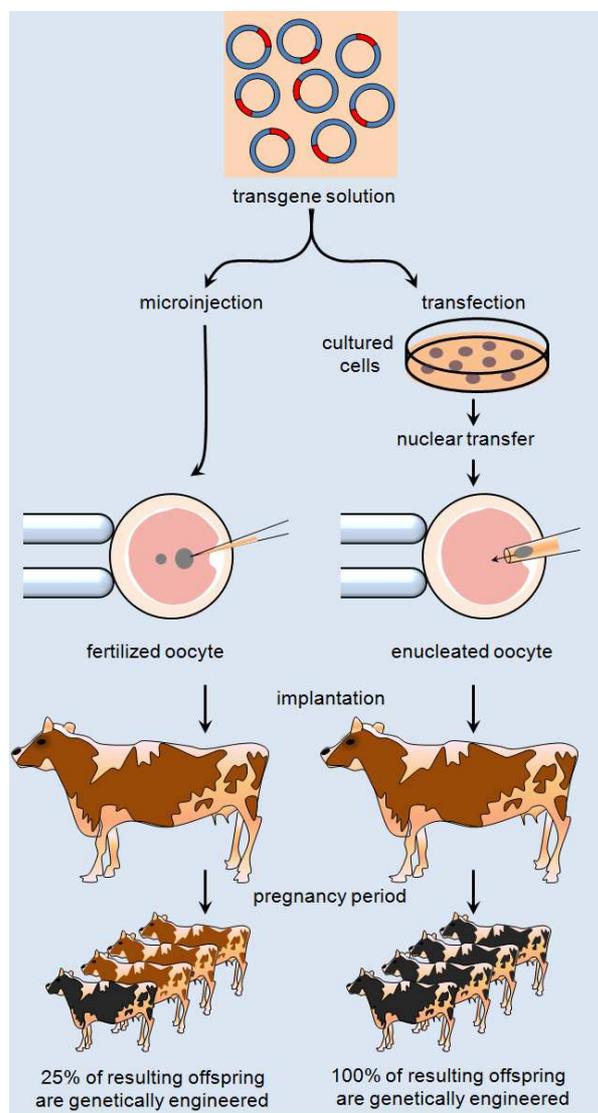
After mixing the genetically engineered DNA (the DNA that inserted into the suitable gene cloning vector) with these spheres, its introduced into the recipient (diseased cells) cells either by incubating these complexes with the diseased cells (*in vitro*), or by direct injection of these complexes into the organisms body (*in vivo*). When the liposome is fused with the cell membrane, it releases the DNA into the cytoplasm. By an unknown mechanism, the vector DNA is transported into the nucleus where the

In performing nuclear transfer, the nucleus is first removed from an unfertilized egg (oocyte) taken from an animal soon after ovulation. This is accomplished by using a dedicated needle to pierce through the shell (zona pellucida) to draw out the nucleus under a high power microscope. The resulting cell, now devoid of genetic materials, is an enucleated oocyte. In the next step, the donor cell carrying its complete nucleus is fused with the enucleated oocyte. The fused cells then develop like a normal embryo, and finally implanted into the uterus of a surrogate mother to produce offspring. One of the most important examples of this technique that found huge advertisement impact is the

cloning of Dolly the sheep, which is a copy of its donor mother animal.

In addition to the efficiency of liposomes, they are not dangerous from the biological side. This, by this feature, may overcome the known viral vectors. Therefore, many types of liposomes are tested on many diseases, and they still under development till now.

9. **Somatic cell nuclear transfer (SCNT) technique;** which is known in its original meaning, the technique that can be used in somatic cell nuclear transfer (SCNT), in its original meaning, can be identified as a technique that can be used to create a genetically identical copy (clone) of an animal (figure 6.28).



**Figure (6.28);** Schematic illustration shows a simple comparison between pronuclear microinjection (left) and somatic cell nuclear transfer (right). In the SCNT, all the generating offspring are transgenic since there is no nucleus except one which represents the donor nucleus. This nucleus is taken from foreign DNA infected somatic cell culture.

In performing nuclear transfer, the nucleus is first removed from an unfertilized egg (oocyte) taken from an animal soon after ovulation. This is accomplished by using a dedicated needle to pierce through the shell (zona pellucida) to draw out the nucleus

under a high power microscope. The resulting cell, now devoid of genetic materials, is an enucleated oocyte. In the next step, the donor cell carrying its complete nucleus is fused with the enucleated oocyte. The fused cells then develop like a normal embryo, and finally implanted into the uterus of a surrogate mother to produce offspring (figure 6.28). One of the most important examples of this technique that found huge advertisement impact is the cloning of Dolly the sheep, which is a copy of its donor mother animal.

Some animals can be generated by SCNT and could be considered as “transgenic”, when only when the donor nuclei is originated from a cell that carries some genetic modification. Therefore, any individual animal generated by SCNT of genetically modified cells will also be defined as “transgenic” since they carry the initial modifications present in the somatic cells from which the donor nuclei have been taken (figure 6.29).



**Figure (6.29).** Transgenic monkeys, which carries the reporter green code for green fluorescent protein.

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